

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
14 February 2002 (14.02.2002)

PCT

(10) International Publication Number  
**WO 02/12528 A2**(51) International Patent Classification<sup>7</sup>: **C12P 7/00**(21) International Application Number: **PCT/US01/24327**(22) International Filing Date: **3 August 2001 (03.08.2001)**(25) Filing Language: **English**(26) Publication Language: **English**(30) Priority Data:  
09/633,294 4 August 2000 (04.08.2000) US  
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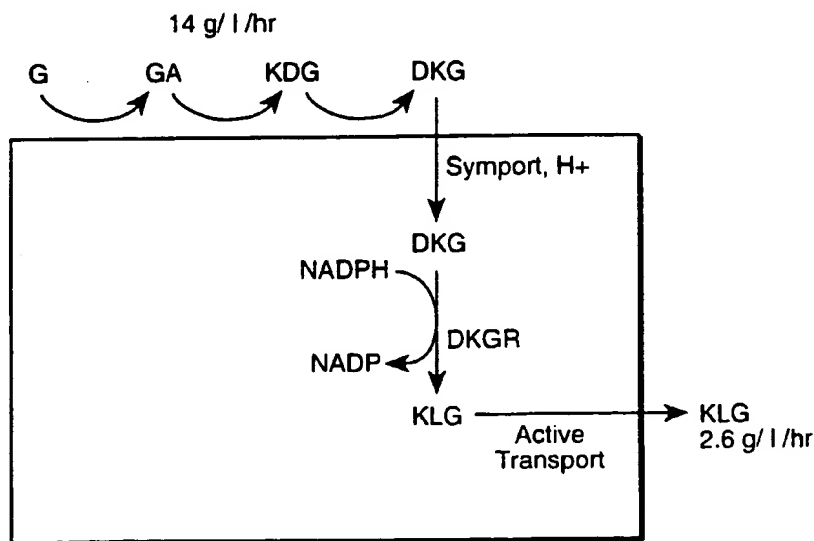
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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SI, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,

[Continued on next page]

(54) Title: **ENHANCED 2-KETO-L-GULONIC ACID PRODUCTION**

Target: 10 g / l / hr

(57) Abstract: A method for enhancing a host cell's biosynthetic production of 2-KL.G is described. Such method comprises selecting a host cell that has an at least partially intracellular synthetic pathway which utilizes 2,5-DKG to produce 2-KL.G; increasing the transport of said 2,5-DKG into said host cell while maintaining the integrity of the host cell; culturing the host cell to produce said 2,5-DKG; and producing 2-KL.G. The transport of the 2,5-DKG is increased by transforming into the host cell DNA encoding for one or more enzymes transporting the 2,5-DKG into the host cell.

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CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Published:**

- *without international search report and to be republished upon receipt of that report*

## ENHANCED 2-KETO-L-GULONIC ACID PRODUCTION

### FIELD OF THE INVENTION

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The present invention generally relates to enhancing the industrial production of 2-KLG and specifically to the overexpressing of genome encoding the protein transporting 2,5-diketoglutarate from the periplasm to the cystolic region of the cell. The present invention provides expression vectors, methods and systems for the enhanced production of a 2-KLG in  
10 microorganisms.

### BACKGROUND OF THE INVENTION

It is generally well known that 2-keto-L-gluonic acid (2-KLG) is readily converted to L-ascorbic  
15 acid (vitamin c) by a one-step chemical procedure in the Reichstein method (Reichstein, T., et al, *Helv. Chim Acta*, 1934, 17 311-328; Reichstein, T., et al, 1933, *Helv Chim Acta*, 16, 561, 1019). There are recombinant microorganisms which express heterologous enzymes to convert a starting substrate to 2-KLG. Recombinant DNA techniques have been used to bioconvert D-glucose to 2-KLG in *Erwinia herbicola* in a single fermentive step (Anderson, S.,  
20 et al *Science* 230, 144-149 (1985)). However, this study is directed to increasing the expression of the 2,5 DKG reductase or other synthetic production without recognizing the importance of substrate transport in the industrial production of the end-product. Indeed, studies by the inventors revealed a minimal increase in the production of 2-KLG as a result of the overexpression of the reductase. Thus there is a need for a means to increase the  
25 industrial production of 2-KLG through biosynthetic pathways utilizing recombinant microorganisms by means other than increasing the expression level of the converting proteins within the cell.

The lipid bilayer of biological membranes is generally impermeable to ions and polar  
30 molecules. These biological membranes compartmentalize a cell, separating different sections of cell from one another. Thus substrates utilized by the cell to synthesize various products as well as metabolites utilized by the cell for generating energy or growth may be separated from the synthetic and/or catabolic reactions which utilize them. With respect to product synthesis, different synthetic pathways or portions thereof, can be found in different  
35 portions of the cell. Some oxidative reactions can occur outside of the cytosol. For example, membrane bound proteins can be used to oxidize a carbon source to another intermediate.

Cystolic reactions or pathways, for example some reductions or dehydrogenations, can also be utilized to convert a substrate or intermediate into another product. When the substrate and the synthetic machinery are on opposite sides of a membrane, production of the desired end-product may require translocation of the substrate to the situs of the synthetic reaction to enable its conversion to the desired end-product. Alternatively, end-products generated inside of the cell membrane may require translocation from within the cell. Since the partitioned sections of the cell may have different environmental parameters, e.g., solute, ion, end-product, etc., concentrations, or may require translocation across a normally impermeable barrier, some form of active transport may be required.

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Responsive to these problems, investigation related to increasing the transport of materials across membranes has occurred. Solvents or lyzing agents have been used to rupture the membranes, enabling the crossing of the materials across membrane. However, such methods have adverse effects upon the viability of the host cell. If the synthetic or metabolic pathways are dependent or energized by a host cell's own metabolic or catabolic pathways, such as requiring co-factors such as NADH or NADPH, destroying the viability of the cell halts further or continued synthetic production by the host cell. In addition, while the increase in the transport of glucose or other saccharides has been explored in increasing the growth of the cell (Parker, C., et al, Mol. Microbiol 15(5):795-802 (1995)), altering cellular transport systems to increase the industrial production of chemical end-products or intermediates through biosynthetic pathways utilizing recombinant microorganisms has not been recognized.

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Cornish (J. of Gen. Microbiol., 134:3111-3122 (1988)) discusses the relationship between glucose transport and the production of a succinoglucan exopolysaccharide by *Agrobacterium radiobacter*. Cornish proposed that glucose uptake was a major kinetic control point for succinoglucan production, and that it ought to be possible to obtain even higher rates of succinoglucan production by using recombinant DNA methods to obtain even higher rates of succinoglucan production. However the production rates of Cornish were not on the scale of industrial needs. Furthermore, high levels of energy expended and complex regulatory mechanism involved in transporting glucose could discourage rather than encourage its use.

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Volschenk, H., et al (Nat. Biotechnol. 15:253 (March 1997)) describes the introduction of malate degradation pathways into *Saccharomyces cerevisiae* by the cloning and expression of heterologous DNA encoding the same for the purpose of depleting the malate levels present

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in wine. Volschenk was primarily concerned with the removal of malate from the surrounding medium, not the production of any desired end product on an industrial scale.

Furthermore, the mere knowledge that a transport system is involved does little to guarantee the enhanced production of the desired end product or intermediate. The synthetic machinery may already be saturated and thus an increased presence of the substrate won't necessarily result in the increased production of the desired end product. In addition, increased transport of a substrate that is utilized directly or indirectly as a metabolite in addition to its use as a production substrate may not result in the increased production desired.

Merely increasing the expression level of the enzyme converting the substrate to the desired end product may not result in an increased production of chemical compounds using recombinant microorganisms. There is a need for a means to increase the production of chemical compounds using recombinant microorganisms by means other than increasing the expression level of substrates within the cell.

There is also a need for a means to increase the industrial production of 2-KLG through biosynthetic pathways utilizing recombinant microorganisms by means other than increasing the expression level of the cytosolic reductases in the cell.

## SUMMARY OF THE INVENTION

The capacity of the 2,5-DKG transport of a microorganism may become a limiting factor or bottleneck to a desired 2-KLG production, in particular since 2-KLG production is compartmentalized in the cytosol and requires the transport of 2,5-DKG from its situs of production, extracellular membrane bound pathways. The present invention provides a means for alleviating that bottle neck.

The present invention provides isolated nucleic acid and amino acid sequences for *P.citrea* PE1, PE6, YiaX2, PermA and PermB. The amino acid sequence and nucleic acid sequence for *P.citrea* PE1, PE6, YiaX2, PermA and PermB is shown in FIGS. 1A-1E SEQ ID NOS: 1 and 2.

The present invention also provides improved methods for enhancing a host cell's biosynthetic production of 2-KLG from 2,5-DKG. Accordingly, a method is provided for enhancing a host cell's biosynthetic production of 2-KLG, the method comprising selecting a host cell that has a

synthetic pathway which converts 2,5-DKG to 2-KLG; increasing the transport of said 2,5-DKG into said host cell while maintaining the integrity of the host cell; culturing the host cell to produce said 2-KLG; and producing the 2-KLG. In another embodiment, the step on increasing the transport of said 2,5-DKG into said host cell includes the step of transforming  
5 into said host cell DNA encoding for one or more proteins transporting said 2,5-DKG into said host cell's cytosolic material. The said one or more proteins is selected from the group consisting of YiaX2, PE1, PE6, PrmA and PrmB. The DNA encoding may also be expressed from genomes selected from the group consisting of *yiaX2*, *pe1*, *pe6*, *prmA* and *prmB*. The one or more proteins is capable of hybridizing with SEQ ID NO \_\_\_\_\_. The protein has at least  
10 50%, or 90% identity with SEQ ID NO or SEQ ID No.\_\_\_\_. In another embodiment, the protein comprises a sequence comprising at least 31 residues, said residues comprising a glycine residue which corresponds to glycine 119 of PermA or optionally a tryptophan residue corresponding to W136 of PermA or optionally at least one additional residue selected from the group of a phenylalanine at a position which corresponds to G138 of PermA, a glutamic  
15 acid (E) at a position which corresponds to E141 of PermA, and an arginine (R) at a position which corresponds to R142 of PermA.

The present invention also provides a method for enhancing the transport of 2,5 DKG into the cytosol across the inner cell membrane, the method comprising selecting a host cell; and  
20 transforming into said host cell DNA encoding for one or more proteins transporting of 2,5 DKG into said host cell. In one embodiment, the host cell is selected from the group consisting of bacteria and yeast. Preferably the host cell is selected from the group consisting of *E. coli*, *Pantoea*, and *Klebsiella*.

25 The present invention provides a method for enhancing the transport of 2,5-DKG into the cytosol across the inner cell membrane by the steps of selecting a host cell and transforming into the host cell DNA encoding for one or more proteins transporting of 2,5 DKG into said host cell

### 30 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the DNA and amino acid sequencing of YiaX2 of *Klebsiella oxytoca*; PE1 (environmental permease); PE6 (environmental permease); PermA of *Pantoea citrea*; PrmB of *Pantoea citrea*; YiaX2 of *Pantoea citrea*.

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FIG. 2 is a flow diagram showing the synthetic pathway for the production of ascorbic acid precursor 2-KLG from glucose.

FIG. 3 is a diagram showing the synthetic pathway of the ascorbic acid precursor 2-Keto-L-gulonic acid (2-KLG).

FIG. 4 is a flow chart showing the various synthetic pathways that glucose can follow to get to 2-KLG. Boudrant, J., Enzym Microb. Tech., 1990, 12, 322-329

FIG. 4A is a diagram showing the synthetic pathways of D-sorbitol to 2-KLG showing the cellular location of the reactions relative to other reactions within the pathway and the transport of substrates across cell membranes. Saito, Y, et al Biotechnol. Bioeng. 58(2/3):305-315 (1998).

FIG. 5 shows the synthetic pathway of D-glucose (G) to 2,5-DKG to 2-KLG, showing the location of the reactions relative to other reactions within the pathway and the transport of the respective substrates across the cell membrane.

FIGS. 6 is a line graph comparing the DKG transport rate with the KLG production rate showing the amount of 2,5-DKG uptake (nmoles/OD 600) v. time (-♦- = fructose feed 139-2a,  $0.0486x + 0.67$ ; -▲- = glucose feed 139-2a,  $y = 0.0497 + 0.5877$ ; -●- = seed flask 139-2a,  $y = 0.0075x + 0.0569$ ).

FIG.7 is a schematic of the yia operon of ascorbic acid catabolism in *Klebsiella oxytoca*.

FIG. 8 is a graph showing the amount of 2,5 DKG uptake (nmol) by *Klebsiella oxytoca* measured by the silicone oil transport assay (-♦- = Tester + isopropyl β-D-Thiogalactopyranoside [IPTG]) ; -▲- = ΔyiaX2 + IPTG); -■- = Tester – IPTG)

FIG. 9 is a schematic drawing showing the selection design to close permeases from *P. citrea*, *K. oxytoca* and environmental sources.

FIG. 10 is a bar graph showing 2,5-DKG uptake activity in *K. oxytoca* strains (YiaX2, pcp1, pcp10, pcp32, pK1, Environmental #1; and Environmental #6).

FIGS. 11 is a bar graph showing 2,5-DKG uptake assay of shaker flask having various DKG permeases (139-2A, 139-2A + PCP32; 139-2A + PCP10; 139-2A + PK1; 139-2A+ PCP1) and 139-2A + PE6.in the same plasmid construct (pBCL 1920) measuring the DKG Uptake rate (g/l/hr) at 28 degrees C.9A-9B .

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FIG. 12 is a line graph showing the specific productivity increase with overexpressed DKG permease/ Spec Production rate (g/L/hr) (-◇ - = wild-type; -x- = WT, pmA)

FIG. 13 is a schematic drawing of the PermA transporter in a membrane surface. The putative membrane spanning domains numbered I-XI. The positions of the conserved residues are indicated in bold. N is the amino-terminus and C is the carboxyl-terminus. Putative membrane-spanning domains of *Pantoea citrea* Permease A (SEQ ID:\_\_), were deduced using the tool available to <http://sosui.proteome.bio.tuat.ac.jp/sosui/frame0.html>

FIG. 14 is a conserved amino acid sequence corresponding to residues G119s through 142.

## DETAILED DESCRIPTION

### Definitions

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#### Transporter definitions

Bacterial channel transporters refers to those transporters generally in the TC classification of #1.A (Saier, M., et al., 1998, *Advances in Microbial Physiology* (Poole, R.K., ed.) pp. 81-136, Academic Press, San Diego, CA.). ("TC" stands for "Transport Council", a classification system which takes into consideration the phylogenetic aspects of the transporter.) These generally transport substrates, ions or other material via an energy independent facilitated diffusion mechanism employing a transmembrane pore.

Primary transporters refers to those transporters generally in the TC classification of (TC #3.A)(Saier, M., et al, 1998), and are those that utilize chemical energy, typically in the form of ATP hydrolysis as a mode of energy coupling for the active uptake and transport extrusion of substrates.

Group translocation systems refers to transporters in the TC classification of TC #4.A. (Saier, M., et al, 1998) are transporters that concomitantly transport and phosphorylate their substrates during transport. The members of this category generally are part of the bacterial



specific phosphotransferase system (PTS) and are characterized by the coupling to the oxidation of phosphoenol pyruvate (PEP) utilization.

Secondary transports refers to those transporters generally in the TC classification of #2.A, (Saier, M., et al, 1998) those that generally use chemiosmotic energy, for instance in the form of a proton gradient, to provide energy to transport the substrate, ions or end products across the membrane.

Major facilitator superfamily (MFS) refers to secondary transporters that are generally in the TC classification of #2 (Saier, M., et al, 1998).

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A transporter refers to any macromolecule that allows the translocating of a chemical compound across a cell membrane and into or out from a cell or cellular compartment. Transporters are also known or referred to as permeases. While not being limited to a specific theory, it is thought that the transporter is a protein that interacts with a membrane, with portions of the protein extending from the outer surface of the membrane, through the membrane, and from the inner surface of the membrane.

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Active transport refers to transport that is coupled with an expenditure of energy, for example the hydrolysis of adenosine tri-phosphate (ATP) or phosphoenolpyruvate (PEP).

An anion/cation symporter refers to a transporter that utilizes an chemiosmotic gradient to transport the substrate across the membrane (TC class 14). They are also referred to as substrate/H<sup>+</sup> symporters.

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**TMS refers to transmembrane spanning domains**

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Pathway definitions

Cytoplasmic refers to being within the inner cell membrane.

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Exogenous substrate refers to a material, found on the opposite side of the separating membrane from the synthetic reaction, e.g., outside of the inner cell membrane when the substrate is to be converted by an intracellular synthetic pathway or an intracellular portion of a synthetic pathway to the desired end product or intermediate.

Extracellular or outside the inner cell membrane refers to cell locations on the opposite side of a membrane from the cytoplasm, including, but not limited to the periplasm.

Inner cell membrane refers to the barrier that separates the cytoplasm from the periplasm.

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Membrane refers to a lipid bilayer that is intrinsically impermeable to the substrate.

Intracellular refers to the portion of the cell on the side of the membrane that is closest to or of the cytosol. Intracellular also includes cystolic.

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Intracellular reaction refers to a synthetic reaction or bioconversion located within the cystolic cell material, i.e., material enclosed inside of the inner cell membrane.

Rate limiting step refers to the step within the 2-KLG biosynthetic pathway, where an increase in the conversion across that step results in an increase in the production of 2-KLG.

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Enhancing the production refers to increased titer (total amount) of the desired intermediate, end-product or precursor of a synthetic reaction, generally measured by an increase in the gm/l/hour obtained through the process. It may also refer to an increase in the rate at which the desired products are made, generally measured in g/l per unit time. of the recombinant production, wherein the amount of end-product, intermediate or precursor produced increases as a result of the transforming of DNA encoding the at least one protein increasing the transport of the substrate across a membrane in the presence of the overexpressed transporter.

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**A substrate refers to 2,5-DKG that is bioconverted by a synthetic reaction, the cytosolic reaction situs being separated from the substrate by a membrane.**

Synthetic reaction refers to the recombinant bioconversion of a substrate to an intermediate or an end-product.

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2,5-DKG reductase refers to a protein which is capable of catalyzing the conversion of 2,5-DKG stereoselectively to 2-KLG.

2,5-DKG transporter refers to a protein which is capable of transporting the 2,5-DKG across the inner cell membrane for conversion to 2-KLG by a 2,5-KLG reductase.

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## Expression definitions

Promoters refers to DNA elements that guide the RNA polymerase to start the transcription of a gene at the appropriate site to generate a messenger RNA capable of forming a polypeptide once it is translated by the translational machinery of the cell.

An "upstream activating sequence" is a binding position for a positively-acting DNA binding regulator. As indicated by its name, the upstream activating sequence is upstream of the transcription start site and is a nucleic acid.

Regulatory regions refers to regions on the DNA that modulate the expression of genes. One mechanism for this modification is that some regulatory regions serve a binding sites for proteins (also known as repressors). Once bound, a repressor interferes with the ability of RNA polymerase to transcribe a gene.

An expression system includes one or more proteins and/or nucleic acids which, when acting together, can increase the expression of a protein in a host cell. The expression system can be encoded on one or more plasmids and may or may not be on the same plasmid as the gene encoding the protein of interest.

The phrase "functionally linked" or "functionally coupled" means that the regulating elements (DNA or protein) interact physically in order to exert their function. This can be a protein/protein, DNA/protein or a DNA/DNA interaction. For example, the DNA binding regulator interacts with the promoter but genes encoding them may be at different sites on the chromosome. As such, the genes encoding the elements can be on different plasmids from each other and from the gene encoding the protein of interest and still work together to regulate expression of the protein.

Commonly, when describing proteins and the genes that encode them, the term for the gene is not capitalized and is in italics, i.e., *permA*. The term for the protein is generally in normal letters and the first letter is capitalized, i.e., *PermA*.

## Organism definitions

"Bacteria" include microorganisms of the class Schizomycetes. Bacteria can be either Gram-negative or Gram-positive. Gram-negative bacteria include members of the genera Escherichia, Hemophilus, Klebsiella, Proteus, Pseudomonas, Salmonella, Gluconobacter, Acetobacter, Yersenia, Shigella, Vibrio, Acinetobacter, Pantoea and Serratia. Gram-positive  
5 bacteria include members of the genera Bacillus, Clostridium, Staphylococcus, Streptomyces, Lactobacillus and Lactococcus.

Gram-negative bacteria can be pantoeans which are strains that are members of the genus Pantoeas. A preferred bacterial is Pantoea citrea. Pantoea citrea is also sometimes referred  
10 to as Erwinia herbicola or Acetobacter ceremius.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

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#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One embodiment of the invention is directed to a method of transforming a host cell with a plasmid that includes the nucleic acid encoding the expression system. Another embodiment  
20 of the invention is directed to a method of transforming a host cell with a plasmid that includes DNA encoding for one or more proteins increasing the transport of the substrate across the membrane. A host cell is a cell into which a plasmid of the present invention can be inserted through, for example, transformation. The host cell is preferably a bacteria and more preferably in the group of Pantoea, Escherichia; Klebsiella or Bacillus.

25 In another embodiment, if regulating elements are incorporated, such elements of the expression system are from E. coli and B.subtilis. In one embodiment, the host cell is preferably a Gram-negative bacteria. In another preferred embodiment, the host cell is a Pantoea. The same host cell can be transformed with a further plasmid that includes a nucleic acid that encodes one or more transporters. Preferably, the transporters are encoded  
30 MFS transporters, more preferably anion/cation symporters. Exemplary transporters include those encoded or expressed to *yiaX2*, *permA*, *perm B*, *pe6*, *pe1* from Pantoea citrea or Klebsiella oxytoca and heterologous sources.

- 11 -

The present invention provides novel methods for enhancing a host cell's biosynthetic production of a 2-KLG, by increasing the transport of 2,5-DKG to ameliorate the bottleneck to pathway synthesis and the production of desired end-products, in particular when the transporters are recombinantly introduced and overexpressed by the host cell.

- 5 One embodiment of the invention is directed to a method of transforming a host cell with a plasmid that includes the nucleic acid encoding an expression system. A host cell is a cell into which a plasmid of the present invention can be inserted through, for example, transformation. The host cell is preferably a bacteria. In one embodiment, the host cell is preferably a Gram-negative bacteria. In another preferred embodiment, the host cell is a
- 10 pantoen. Preferably, the host cell is *Pantoea citrea* and, if regulating elements are incorporated, such elements of the expression system are from *Pantoea*. The same host cell can be transformed with a further plasmid that includes a nucleic acid that encodes one or more transporters. Preferably, the transporters are encoded or expressed from *yiaX2*, *permA*, *perm B*, *pe6*, *pe1* from *Pantoea citrea*.

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#### Synthetic Reaction

- The present invention provides for the increased transport of 2,5-DKG across a membrane to enhance the production of 2,5-DKG from 2-KLG. The increased transport provides for translocation of the 2,5-DKG across a membrane separating 2,5-DKG from the cellular
- 20 location of the reduction reaction (Figs. 2 and 3).

- The present invention is particularly useful in conjunction with ascorbic acid intermediate synthesis, for example the conversion of 2,5 DKG to 2-KLG; the conversion of sorbose or sorbitol to 2-KLG via sorbosone; the reduction of 5-keto-D-Gluconic acid (5-KDG) to L-idonic acid; and the reduction of 5-Keto-D-Gluconic acid to L-gulonic acid . Each of these pathways
- 25 is characterized by a portion of the synthetic pathway, a synthetic reaction, that resides within the cytoplasm, e.g. the reduction of 2,5-DKG by 2,5 DKG reductase; the reduction of L-sorbosone to 2-KLG by a sorbosone dehydrogenase; the reduction of 5-keto-D-Gluconic acid (5-KDG) to L-idonic acid by 5-KDG dehydrogenase; and the reduction of 5-Keto-D-Gluconic acid to L-gulonic acid by 5-KDG reductase. These pathways are also characterized by the
- 30 necessity of transporting the substrate, e.g., 2,5-DKG; L-sorbosone, etc. across the membrane for bioconversion by the synthetic reaction residing in the cytoplasm.

The substrate is generally one that can not pass through the membrane efficiently without some sort of active transport mechanism. Preferably these can include, but are not limited to ascorbic acid intermediates (2,5-DKG, sorbosone). In addition, the substrate is a material that is transported for synthetic use on an industrial scale and generally not for metabolic use by the host cell. Industrial scale refers to the titer and volumetric productivity of being greater than 1 gm/liter/hour, preferably greater than 2 g/l/h, more preferably greater than 3 g/l/h and still more preferably greater than 5 g/l/h. In another embodiment, the productivity titer is between 2 and 14 gm/l/hour, preferably between 3 and 12 g/l/h, and still more preferably between 5 and 10 g/l/h to be an economically viable industrial production process. Especially preferred substrates include 2,5-DKG; and sorbosone. The inventive aspects of the present invention are especially useful in these embodiments,

A reaction especially useful in the practice of this invention is the transport of 2,5-DKG across the inner cell membrane for the cytosolic reduction of the same to 2-KLG by the cytosolic dehydrogenase 2,5-DKG reductase. Boudrant, J. (1990) Enzyme Microb, Technol., 1990:322-329) 2,5-DKG is converted from 2-keto-D-gluconate (2-KDG) by membrane bound 2-ketogluconate dehydrogenase. 2-KDG is converted from glucose through oxidation of D-gluconate (GA). The inventors recognize that the transport of 2,5-DKG across the inner cell membrane to the site of the cytosolic reduction to 2-KLG could be achieved by the DNA encoding an increase in the transport of 2,5-DKG.

Another reaction especially useful in the practice of this invention is the transport of sorbosone, an intermediate in the production of 2-KLG through sorbose, sorbitol (Saito, Y, et al, Biotechnol. Bioeng. 58(2/3):309-315 (19987). The conversion of sorbitol and /or sorbose to sorbosone is a step in the pathway of converting sorbitol or sorbose to 2-KLG (Boudrant, J., 1990; Saito (1997)). The following is a discussion of engineering of sorbosone transporters according to the present invention.

As described Saito, the pathway of D-sorbose to 2-KLG includes the oxidation of L-sorbose to L-sorbosone by L-sorbose dehydrogenase (SDH), followed by the oxidation of L-sorbosone to 2KLG by L-sorbosone dehydrogenase. One recombinant host cell has been described which converts D-sorbitol to L-sorbosone by membrane bound dehydrogenases (Saito, Y., et al (1997)). L-sorbosone is then transported from the periplasm for reduction by L-sorbosone-dehydrogenase in the cytoplasm. Overexpression of the sorbosone transporter to facilitate the transport of the sorbosone intermediate to the pathway for conversion to 2-KLG is perceived as having a beneficial effect.

An alternative pathway of D-glucose to 2-KLG includes the **oxidation** of D-gluconic acid to 5-Keto-D-Gluconic acid (5KDG) which in turn is reduced to L-idonic acid (IA) or L-gulonic acid for oxidation to 2KLG. (Boudrant, J. (1990)). Transport of 5-Keto-D-Gluconic acid into the cytosol for reduction by keto-reductase could also be facilitated by the overexpression of the  
5 5KDG transporter.

In another embodiment, the synthetic reaction may be extracystolic or located outside the membrane relative to the substrate. The end-product of the first reaction may be the an intermediate substrate for a second reaction on the opposite side of a membrane. For example, in the synthesis of 2-KLG from D-sorbitol in *Gluconobacter oxydans* T-100 from  
10 Japanese persimmon (FERM BP-4188), the conversion of D-sorbitol to L-sorbose by cytosolic L-sorbitol dehydrogenase results in an intermediate that is transported out of the cytoplasm, across the cell membrane for conversion to L-sorbose by the membrane bound L-sorbose dehydrogenase. Thus the inventors contemplate increasing the transport of the cytosolic intermediate, a substrate, from the cytosolic side of the inner membrane across the  
15 membrane to outside the membrane for subsequent conversion. Saito, Y., (1997)

While a preferred embodiment includes the synthetic reaction or the pathways including the same as being within a single organism, having separate reactions in separate organisms is also contemplated by the inventors. For example, in a mixed culture system for the production of 2-KLG from glucose, the conversion of glucose to an intermediate 2,5-DKG,  
20 may occur within one organism (Acetomonas, Acetobacter, Gluconobacter or Erwinia) while the conversion of that intermediate to the desired ascorbic acid intermediate 2-KLG occurs within the second organism (Brevibacterium, or Corynebacterium) see US Patent No. 3,963,574 to Sonoyama (1976). See also Hoshino, US Patent No. 5,312,741.

Therefore the synthetic reaction may generate an intermediate that itself may be converted at  
25 another cellular location separated by a cell membrane. The end-product, in this embodiment, may be an intermediate substrate for a subsequent reaction.

#### Methods for Determining the Rate limiting step of the

One reason for the effect of the increase transport of 2,5-DKG across the inner cell membrane to the cystolic cell location in the production of 2-KLG is because the inventors recognized that  
30 it is the rate limiting step of the conversion of 2,5 DKG to 2-KLG. The determination of whether such a step is a rate limiting step was determined by analyzing the pathway, assessing the production of each step and altering the amount of 2,5-DKG available for

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conversion by the pathway to ascertain whether such increased presence of 2,5-DKG results in an increase in the overall production of the pathway. Upon determining that increasing the presence of 2,5-DKG enhances the production of 2-KLG, increasing the transport of the substrate will enhance the production of 2-KLG.

- 5 The determination of the rate limiting step can be ascertained by comparing the productivity of the microorganism. One method for determining the rate limiting status of the pathway portion is to compare the intermediate productivity at various points of the pathway, before and after increasing the presence of a particular chemical compound. Increasing the presence of the reductase by overexpression of the DNA encoding the 2,5-DKG reductase did not result  
10 in an increased production of 2-KLG. Fig. \_\_\_\_\_. However, increasing the amount of 2,5-DKG present in the cytosol resulted in an increased production of 2-KLG. Thus the inventors recognized that increasing the amount of 2,5-DKG was a rate limiting step in the production of 2-KLG.

- One method for determining the rate limiting status of the pathway portion is to compare the  
15 intermediate productivity at various points of the pathway, before and after increasing the presence of a particular bioconverter. **If there is no increase in the production of the end-product despite increased presence of an intermediate or the overexpression of the converting pathway, the step may not be rate limiting, and thus overexpression of the particular enzyme effecting the synthetic reaction may not result in an enhanced**  
20 **production.** The amounts of the individual intermediates can be determined by various indirect or direct means. Indirect means includes measuring the consumption or production of respiratory parameters, e.g. carbon dioxide production, oxygen consumption, by in-line measurements, such as gas partial pressures. Direct measurement of the intermediates can be achieved by various analytical techniques known in the art as described by Lazarus, *Analyt.*  
25 *Biochem* 157, 360-366 (1986) and references cited therein, which are incorporated by reference herein, including, but not limited to paper, gas, liquid and thin-layer chromatography as well as chemical and enzymatic assays. High performance liquid chromatography methodologies, especially as set forth in Lazarus, 1986, are especially helpful. One method used by the inventors includes the Waters 2690 HPLC and Waters 410 differential  
30 refractometer, settings, using a 50mM acetate buffer, 1 ml/minute flow rate, as the eluting medium and Dyonex Ionpac AS-10 ion-exchange column (4x250 mm) for separating and quantifying the chemical compounds present.

Another method used by the inventors to determine the purity of the 2-KLG produced in the broth was by total carbon analysis.



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Thus in one embodiment, the transport activity can be measured in any cell in which the substrate can be converted to a product, by measuring production of the product in the presence of extracellular substrate. For example, in a cell naturally expressing, or recombinantly expressing, a 2,5-DKG reductase, intracellular 2,5-DKG is converted to 2-KLG. The ability of the bacterial cell to produce 2-KLG when provided with extracellular 2,5-DKG, upon expression of a 2,5-DKG permease, is a measure of the ability of the expressed permease to transport 2,5-DKG into the cell, and is thus a measure of its 2,5-DKG permease activity. Intracellular 2-KLG can be detected, for example, using HPLC or other sensitive detection methods known in the art. Other metabolic products of 2,5-DKG can also be detected, by similar methods.

#### The 2,5-DKG transporter

There are four distinct types of functionally characterized transport systems based upon mode of transport and energy coupling mechanisms. The first are bacterial channel proteins (TC#1.A), which transport via an energy independent facilitated diffusion mechanism utilizing a transmembrane pore. A second transport system, the facilitators and/or secondary transporters (the second class, TC#2.A), represent the largest category of transporters. A third group ATP driven primary active transporters constitute a use ATP hydrolysis as a mode of energy coupling for the active uptake and or extrusion of solutes. The last group consist of group transports that phosphorylate their substrates during transport (TC #4.A).

Of the secondary families, the largest of the class II families are the major facilitator superfamily (MFS) and the amino acid polyamine choline (APC) family (reizer et al. These secondary transporter families can be further divided into three groups (1) proton motive force (pmf driven), (2) sodium motive force (smf)-driven, and (3) other ion or solute driven exchangers. These transport systems catalyze uni, anti and/or symport of solutes. Secondary active transporters have been identified in *E.coli*, *H.influenzae*, *H. pylori*, *B. subtilis*, *M. genitalium*, *Synechocystis*, *M.Jannaschii* (Paulsen et al 1998 *J. Mol. Biol.* 277:573-592). All members of this category are part of the bacterial specific phosphotransferase system (PTS). Secondary transporters are typically polytopic membrane proteins, frequently with 12 TMS with most primary carriers, a chemical form of energy drive the group translocation, be it ATP-dependent systems as most ATP-binding cassette (ABC) superfamily members are, or PTS, which use PEP as the phosphoryl donor for sugar uptake and phosphorylation. Secondary transporters differ from primary (ABC transporters) in that the primary transporters use ATP, taking energy away from the cell. In addition, the use of ABC

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transporters requires a more complex transporter system, one that comprises two hydrophobic integral membrane domains, and two ATP-binding domains (Hosie, et al *Molecul. Microbiol* (2001) 40(6), 1449-1459. Those ABC transporters responsible for the uptake of solutes also require the presence of a solute-binding protein (SBP). Thus genetic engineering  
 5 of an improved ABC transport system would require the expression and transformation of a more complex nature than one of a secondary transporter.

In one embodiment, the DNA encoding the at least one protein for increasing the transport of the substrate across the inner cell membrane is selected from *Acetobacter*, *Pseudomonas*, *Bacterium*, *Cyanococcus*, *Micrococcus*, *Brevibacterium*, *Arthrobacter*, *Staphylococcus*,  
 10 *Bacillus*, *Corynebacterium*, *Acetomonas*, *Gluconobacter* and *Erwinia*. Preferred organisms are selected from the group consisting of *E. coli*, *Pantoea* and *Klebsiella*. *Pantoea* is the most preferred is organism to use as a host cell.

Especially useful transporters include those encoded by *yiaX2* (from *Klebsiella oxytoca*), *pe1*  
 15 and *pe6* (from environmental sources), and *yiaX2*, *permA* and *permB* from *Pantoea citrea*. *yiaX2*, *permA* and *permB* genes can be found in a variety of bacteria such as *Erwinia*, *Acetobacter*, *Gluconobacter*, *E. coli*, *Agrobacter*, *Yersenia*, *Salmonella*, *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Bacillus*, *Citrobacter*. Species include as *Yersenia pestis*, *Yersenia pseudotuberculosis*, *Salmonella*  
 20 *typhimurium*, *Pseudomonas aeruginosa*. *Streptomyces coelicolor*

The present invention provides *YiaX2* polynucleotide, *PermA* polynucleotide, *PermB* polynucleotide, *Pe1* polynucleotide and *Pe6* polynucleotide which may be used as DNA encoding the at least one enzyme increasing the transport of the substrate across the  
 25 membrane in the host cell. **The polynucleotide sequences for *YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* can be determined from FIGS. 13 and 14 which show the amino acid alignment of *P. citrea YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* with the *Klebsiella YiaX2*.**

The present invention encompasses *YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* polynucleotide  
 30 homologs encoding transporters *YiaX2*, *PermA*, *PermB*, *PE1* and *PE6*, respectively, whether encoded by one or multiple polynucleotides which have at least 65%, 70%, 80%, or at least 90% or at least 95% identity to *P. citrea YiaX2*, *PermA*, *PermB*, *PE1* and *PE6*, respectively as long as the homolog encodes a protein that is able to function by modulating transport, preferably increasing transport, of a substrate in a microorganism. As will be understood by  
 35 the skilled artisan, due to the degeneracy of the genetic code, a variety of polynucleotides, i.e., *YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* polynucleotide variants, can encode the *Pantoea*

citrea transporters on factors YiaX2, PermA, PermB, PE1 and PE6 . The present invention encompasses all such polynucleotides.

Microorganism polynucleotide homologs of *P. citrea*, *Klebsiella oxytoca* and environmental  
5 isolates YiaX2, PermA, PermB, PE1 and PE6 transporters can be identified through nucleic  
acid hybridization of microorganism nucleic acid of either genomic or cDNA origin. The  
polynucleotide homolog sequence can be detected by DNA-DNA or DNA-RNA hybridization  
or amplification using probes, portions or fragments of the DNA encoding the at least one  
polynucleotides transporting the 2,5-DKG into the host cells cytosolic material . Accordingly,  
10 the present invention provides a method for the detection of YiaX2, PermA, PermB, PE1 and  
PE6 polynucleotide homologs which comprises hybridizing a nucleic acid sample with part or  
all of a nucleic acid sequence from YiaX2, PermA, PermB, PE1 and PE6.

Also included within the scope of the present invention are YiaX2, PermA, PermB, PE1 and  
15 PE6 polynucleotide sequences that are capable of hybridizing to part or all of the YiaX2,  
PermA, PermB, PE1 and PE6 nucleotide sequence of FIGS. 1 under conditions of  
intermediate to maximal stringency. Hybridization conditions are based on the melting  
temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987,  
Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press,  
20 San Diego CALIF.) incorporated herein by reference, and confer a defined "stringency" as  
explained below.

"Maximum stringency" typically occurs at about  $T_m - 5^\circ\text{C}$ . ( $5^\circ\text{C}$ . below the  $T_m$   
of the probe); "high stringency" at about  $5^\circ\text{C}$ . to  $10^\circ\text{C}$ . below  $T_m$ ;  
25 "intermediate stringency" at about  $10^\circ\text{C}$ . to  $20^\circ\text{C}$ . below  $T_m$ ; and "low  
stringency" at about  $20^\circ\text{C}$ . to  $25^\circ\text{C}$ . below  $T_m$ . As will be understood by those  
of skill in the art, a maximum stringency hybridization can be used to identify or detect  
identical polynucleotide sequences while an intermediate or low stringency hybridization can  
be used to identify or detect polynucleotide sequence homologs.

30 The term "hybridization" as used herein shall include "the process by which a strand of nucleic  
acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of  
Biotechnology, Stockton Press, New York N.Y.).

35 The process of amplification as carried out in polymerase chain reaction (PCR) technologies  
is described in Dieffenbach C W and G S Dveksler (1995, PCR Primer, a Laboratory Manual,

Cold Spring Harbor Press, Plainview N.Y.). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from the *PermA* nucleotide sequence of FIGS. 1A-1E, preferably about 12 to 30 nucleotides, and more preferably about 20-25 nucleotides can be used as a probe or PCR primer.

5

#### Amino Acid Sequences

The *P. citrea PermA* polynucleotide as shown in Fig 1 encodes *P. citrea PermA*. The *P. citrea permA* gene specifies one protein of 436 residues with a calculated molecular mass of 47801.94 Daltons. The average hydrophobicity was 0.62 and the isoelectric point was 9.24. The *permA* protein is an integral membrane protein with 11 putative transmembrane helices. These domains show significant sequence similarity to other known transporter proteins from other organisms, the highest similarity being found with KDG transporter proteins from the *Pseudomonas*. The stretch of 31 residues as shown in Fig 8B, shows the highly conserved segment corresponding the residues of the *P. Citrea PermA* transport protein to a glycine (G) at 119 of *PermA*, a glutamic acid (E) at 122, a phenylalanine (P) at 127, a tryptophan (W) at 136, a phenylalanine at 138, a glutamic acid (E) at 141 and an arginine (R) at 142. This highly conserved segment is consistent with the conserved residues of the anion:cation symporter (ACS) family (Pao, S.S., 1998) Table 3 and 4 supra. Fig 8B shows the conserved regions corresponding to residues 119 through 141 of *PermA*. Putative membrane-spanning domains (I-XI) are indicated in gray shading. The membrane-spanning domains of Figure 8B were determined by SOUCI program.

Transporters within the scope of the present invention include those encoded by *yiaX2*, *permA*, *pe1*, *pe6*, and *permB* from *Pantoea citrea*, *Klebsiella oxytoca* and environmental sources. *YiaX2*, *permA*, *pe1*, *pe6*, and *permB* genes can be found in a variety of species of bacteria such as *Erwinia*, *acetobacter*, *gluconobacter*, *E. coli*, *Agrobacter*, *Yersinia*, *Samonella*, *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, *micrococcus*, *staphylococcus*, *pseudomonas* and *Bacillus*.

30

The present invention provides *YiaX2* polynucleotide, *Perm A* polynucleotide, *PE1* polynucleotide, *PE6* polynucleotide, and *PermB* polynucleotide which may be used as DNA encoding the at least one proteins increasing the transport of 2,5-DKG across the membrane in the host cell. The polynucleotide sequences for *YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* can be determined from FIGS. which show the amino acid alignment of *P.citrea YiaX2*, *PermA*, *PermB*, *PE1* and *PE6* with the *Kielbsiella YiaX2*.

35

The present invention encompasses YiaX2, PermA, PermB, PE1 and PE6 polynucleotide homologs encoding transporters YiaX2, PermA, PermB, PE1 and PE6, respectively, whether encoded by one or multiple polynucleotides which have at least 65%, 70%, 80%, or at least 90% or at least 95% identity to *P. citrea* YiaX2, PermA, PermB, PE1 and PE6, respectively as long as the homolog encodes a protein that is able to function by modulating transport of a substrate in a microorganism. As will be understood by the skilled artisan, due to the degeneracy of the genetic code, a variety of polynucleotides, i.e., YiaX2, PermA, PermB, PE1 and PE6 polynucleotide variants, can encode the *Pantoea citrea* transporters or factors YiaX2, PermA, PermB, PE1 and PE6. The present invention encompasses all such polynucleotides.

Microorganism polynucleotide homologs of *P. citrea*, *Klebsiella oxytoca* and environmental isolates YiaX2, PermA, PermB, PE1 and PE6 transporters can be identified through nucleic acid hybridization of microorganism nucleic acid of either genomic or cDNA origin. The polynucleotide homolog sequence can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments disclosed in Figs. [what number]. Accordingly, the present invention provides a method for the detection of YiaX2, PermA, PermB, PE1 and PE6 polynucleotide homologs which comprises hybridizing a nucleic acid sample with part or all of a nucleic acid sequence from YiaX2, PermA, PermB, PE1 and PE6.

Also included within the scope of the present invention are YiaX2, PermA, PermB, PE1 and PE6 polynucleotide sequences that are capable of hybridizing to part or all of the YiaX2, PermA, PermB, PE1 and PE6 nucleotide sequence of FIG. 1 under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CALIF.) incorporated herein by reference, and confer a defined "stringency" as explained below.

"Maximum stringency" typically occurs at about  $T_m - 5^\circ\text{C}$ . ( $5^\circ\text{C}$ . below the  $T_m$  of the probe); "high stringency" at about  $5^\circ\text{C}$ . to  $10^\circ\text{C}$ . below  $T_m$ ; "intermediate stringency" at about  $10^\circ\text{C}$ . to  $20^\circ\text{C}$ . below  $T_m$ ; and "low stringency" at about  $20^\circ\text{C}$ . to  $25^\circ\text{C}$ . below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York N.Y.).

5

The process of amplification as carried out in polymerase chain reaction (PCR) technologies is described in Dieffenbach C W and G S Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview N.Y.). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from the PermA nucleotide sequence of  
10 FIGS. 1, preferably about 12 to 30 nucleotides, and more preferably about 20-25 nucleotides can be used as a probe or PCR primer.

## II. Expression Systems

15 The present invention provides expression systems for the enhanced production and transport of desired heterologous or homologous proteins in microorganisms, including bacteria and yeast.

### a. Coding Sequences

20

In the present invention, the vector comprises at least one copy of nucleic acid encoding a transporter and preferably comprises multiple copies. In a preferred embodiment, the microorganism is *Pantoea*. In another preferred embodiment, the microorganism is *Klebsiella*. In one embodiment, polynucleotides which comprise the *permA* gene are utilized to construct  
25 the vector. These polynucleotide segments can comprise of a greater number of residues than *permA*. For example, *pcp1*, *pcp10* and *pcp32* are nucleotide fragments that are operons or domains of the *Pantoea citrea* genome. These polynucleotide segments are about 9 kilobases (kb), 13 kb and 67 kb respectively. In a preferred embodiment, polynucleotides which encode *P. citrea* *PermA*, *YiaX2*, *PermB*, *PE1*, and/or *PE6*, or fragments thereof, or  
30 fusion proteins or polynucleotide homolog sequences that encode amino acid variants of *PermA*. *YiaX2*, *PermB*, *PE1*, and/or *PE6*, may be used to generate recombinant DNA molecules that direct the expression of *PermA*. *YiaX2*, *PermB*, *PE1*, and/or *PE6*, or amino acid variants thereof, respectively, in gram-positive host cells. In one embodiment, the host cell is selected from the group consisting of *Acetobacter*, *Pseudomonas*, *Bacterium*,  
35 *Cyanococcus*, *Micrococcus*, *Brevibacterium*, *Arthrobacter*, *Staphylococcus*, *Bacillus*, *Corynebacterium*, *Acetomonas* and *Gluconobacter*. Preferred host cells are selected from the

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group consisting of *Escherichia*, *Pantoea* and *Klebsiella*. *Pantoea citrea* and *Klebsiella* are the most preferred organisms to use as a host cell. *Pantoea* is also known as *Erwinia*.

As will be understood by those of skill in the art, it may be advantageous to produce polynucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular host cell (Murray E et al (1989) *Nuc Acids Res* 17:477-508) can be selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

Altered *PermA*, *YiaX2*, *PermB*, *PE1*, and/or *PE6* polynucleotide sequences which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotide residues resulting in a polynucleotide that encodes the same or a functionally equivalent *PermA*, *YiaX2*, *PermB*, *PE1*, and/or *PE6* homolog, respectively. As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring gram positive *PermA*, *YiaX2*, *PermB*, *PE1*, and/or *PE6*.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The encoded protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent gram-positive *PermA*, *YiaX2*, *PermB*, *PE1*, and/or *PE6* variant. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the variant retains the ability to modulate transport, preferably to increase transport. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine, phenylalanine, and tyrosine.

The PermA, YiaX2, PermB, PE1, and/or PE6 polynucleotides of the present invention may be engineered in order to modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-directed mutagenesis to insert new restriction sites to change codon preference, for  
5 example.

In one embodiment of the present invention, a PermA, YiaX2, PermB, PE1, and/or PE6 polynucleotide may be ligated to a heterologous sequence to encode a fusion protein. A fusion protein may also be engineered to contain a cleavage site located between the PermA.  
10 YiaX2, PermB, PE1, and/or PE6 nucleotide sequence and the heterologous protein sequence, so that the PermA, YiaX2, PermB, PE1, and/or PE6 protein may be cleaved and purified away from the heterologous moiety.

#### b. Vector Sequences

15 Expression vectors used in expressing the transporters of the present invention in microorganisms comprise at least one promoter associated with a transporter factor selected from the group consisting of PermA, YiaX2, PermB, PE1, and/or PE6, which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the  
20 wild-type promoter for the selected transporter and in another embodiment of the present invention, the promoter is heterologous to the transporter, but still functional in the host cell.

Additional promoters associated with heterologous nucleic acid encoding desired proteins or polypeptides may be introduced via recombinant DNA techniques. In one embodiment of the  
25 present invention, the host cell is capable of overexpressing a heterologous protein or polypeptide and nucleic acid encoding one or more transporter(s) is(are) recombinantly introduced. In one preferred embodiment of the present invention, nucleic acids encoding the at least one protein or more proteins increasing the transport of the substrate may be stably integrated into the microorganism genome. In another embodiment, the host cell is  
30 engineered to overexpress DNA encoding for one or more proteins increasing the transport of said substrate into said host cell of the present invention and nucleic acids encoding the heterologous protein or polypeptide is introduced via recombinant DNA techniques. The present invention encompasses host cells that are capable of overexpressing other transporters known to those of skill in the art, including but not limited to, those identified in  
35 Tables 1, 2 or 3 or other transporters known to those of skill in the art or identified in the future.



In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also  
5 comprises one or more selectable markers. As used herein, the term selectable marker refers to a gene capable of expression in the gram-positive host which allows for ease of selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to antibiotics, such as, erythromycin, spectinomycin, chloramphenicol and tetracycline. Also provided are embodiments in which a transporter encoded by a nucleic acid  
10 having at least 90% homology with the DNA sequence shown in SEQ ID NOS:[\_\_\_\_\_]. Preferably, the homology is at least 95%, more preferably at least 98%. Homology can be determined by lining up the claimed amino acid or DNA sequence with another sequence and determining how many of the amino acids or nucleotides match up as a percentage of the total. Homology can also be determined using one of the sequence analysis software  
15 programs that are commercially available, for example, the TFASTA Data Searching Program available in the Sequence Analysis Software Package Version 6.0 (Genetic Computer Group, University of Wisconsin Biotechnology Center, Madison, Wis. 53705).

One can screen for homologous sequences using hybridization as described herein or using  
20 PCR with degenerate primers. Chen and Suttle (1995) Biotechniques 18(4):609-610, 612.

Also, in several embodiments of the invention, there are provided nucleic acids that can hybridize with the DNA or fragments thereof, shown in FIGS. and , SEQ ID NOS:, respectively, under stringent conditions. Stringent hybridization conditions include stringent  
25 hybridization and washing conditions as is known to one of ordinary skill in the art. Hybridization and appropriate stringent conditions are described in Sambrook et al. 1989 Molecular Cloning 2d ed., Cold Spring Harbor Laboratory Press, New York.

Methods for Increasing Production of Recombinant Peptides from Host Cells having  
30 transformed DNA encoding transport proteins

A particularly powerful method of increasing the transport of the substrate from one cellular location to another involves the deletion of [metabolic diversions] genomes from the transformed host cell and the concomitant provision of DNA encoding which increases the transport of the desired substrate. This is advantageously achieved by providing to the cell a  
35 deletion-transporter chimera or fusion protein, in which the metabolic diversions of the deleted

portion are minimized, and in which the transporter ability portion is overexpressed. Chemically-fused polypeptides, or shuffled sections of the genome are a possibility, but recombinant proteins are naturally most preferred for use in this manner. The identification of appropriate permease fragments for use in such a chimera has been described herein above.

5

In terms of the transport portions of these fusion proteins, any permease-derived sequence that contains enough primary sequence information to confer transport activity to the chimera will be useful in this context. However, it will often be preferred to use the entire transporter enzyme as this is more straightforward in terms of methodology. Again, one may look to the  
10 extensive information available in various published references in order to assist with the identification of appropriate transporters or fragments thereof.

#### Transformation

The present invention also contemplates augmenting or increasing the capabilities of cells to produce biologically active polypeptides, such polypeptides increasing the transport of a  
15 substrate from a first location of the cell across a membrane to a second location of the cell. This can be accomplished, in some instances, by overexpressing the proteins involved in the transport of a substrate to another cellular location for additional bioconversion, such as a secondary transporter of the anion/cation symporter (Saier, 1988), in one embodiment an anion/cation H<sup>+</sup> symporter. Exemplary symporters that are contemplated by the inventors  
20 include permeases YiaX2, PE1, PE6, prmA and prmB from *Klebsiella oxytoca* and *Pantoea citrea*.

Expression of transporters involved in maintaining the viability and productive qualities of host cells, especially their transport capacity, is important. In the event that the pathways are  
25 NADPH or NADH requiring reactions, the continued viability of the host cells is a necessity for successful continuous production by the desired pathway. Certain considerations and factors can be kept in mind while determining the number of multiple copies or promoters that can be overexpressed while maintaining the viability of the organism. In general, excessive expression of transporters can be detrimental to the cells because the available space to  
30 incorporate the transporter in the membrane is limited. Therefore, very excessive overproduction of one transporter may decrease the incorporation in the membrane of other transporters that may be involved in the transport of other nutrients from the media . Engineering the overexpression of a cell type-specific transcription factor could increase or stabilize the transporter capabilities of engineered host cells.

Stable overexpression of H<sup>+</sup> symporters in bacterial host cells will serve several purposes. It will increase transgene expression under while maintaining the viability of the microorganism. The overexpression of the symporters is simpler than overexpression of ABC transporters  
5 since symporters do not require the extensive encoding for the multiple components of the ABC transporter. Thirdly, by overexpressing a secondary transporter instead of ATP or PTS coupled transports diversion of energy requirements from the host cell is minimized.

10 In one embodiment of the present invention, nucleic acid encoding one or more transporters of the present invention is introduced into a host cell via an expression vector capable of replicating within the host cell. Suitable replicating plasmids for *Pantoea* are described in Sambrook, et al, 1989, Molecular Cloning 2d ed., Cold Spring Harbor Laboratory Press, New York), hereby expressly incorporated by reference, based on the fact that *Pantoea* sustain the replication of the same plasmids that *E.coli*.

15 In another embodiment, nucleic acids encoding one or more transporters is stably integrated into the microorganism genome. Preferred host cells are from the genus *Pantoea*. Another preferred host cell is *K. oxytoca*. Several strategies have been described in the literature for the integration of DNA into the chromosome (see for example Balbas, et al., 1996, Gene  
20 172:65-69; LeBorge, et al, 1998, Gene 223: 213-219)

Transformation of *P.citrea* can be accomplished by the electroporation method, using the protocol developed for *E.coli* (Potter, H., 1988, Anal.Biochem. 174:361-373).

### III. Identification of Transformants

25 After introducing the DNA into the host, the transformants are selected by antibiotic resistance encoded in the vector or in general by selecting for a function coded within the plasmid. Once transformants have been differentiated from non-transformed cells, the presence of the plasmid with an intact structure can be confirmed using standard protocols (Sambrook, et al, 1989)

30 The presence of the YiaX2, PermA, PermB, PE1, and Pe6 polynucleotide sequence can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of the polynucleotide sequence as disclosed in FIG 1. .

### IV. Transport Assays

A preferred method for determining the levels of the ascorbic acid intermediates by HPLC methodology has been discussed supra.

In addition, a wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting specific polynucleotide sequences include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the nucleotide sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway N.J.), Promega (Madison Wis.), and US Biochemical Corp (Cleveland Ohio) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in U.S. Pat. No. 4,816,567 and incorporated herein by reference. Also, recombinant immunoglobulins may be produced as shown in U.S. Pat. No. 4,816,567 and incorporated by reference.

## EXAMPLES

### Example 1

#### Materials and Methods

a. Plasmid, bacterial strain and media: Plasmid pBCL1920, *K. oxytoca*, *P. citrea* 1392A. *P. citrea* 1392A strain is a *P. citrea* variant which is 39140. pD92 is described in stands for a vector which contains DKG reductase gene. U.S. Patent No. 5,376,544. Murphy III medium contained fructose 0.5%, Phosphate 1.6%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2%, Soytone 0.2%, citrate 0.01%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1%, Trace salts in ppm range such as Fe, Co, Mn, Zn and vitamins such as nicotinic acid, folate and B12; M9 medium, 0.9% Phosphat, 0.1% NaCl, 0.1% NH<sub>4</sub>Cl, MgSO<sub>4</sub> 0.0005%, CaCl<sub>2</sub> 0.025%; Fermentation medium Potassium. Phosphat 1%, MgSO<sub>4</sub> 0.15%, glutamate 1.5%, fructose 2.5%, ammonium sulfate 0.1%, and vitamin blend having biotin, thiamin, pyridoxine,

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riboflavin, nicotinic acid, folic acid and B12, and trace metals cocktail solution having iron, Zn, and Mn ions in 10-100 ppm level; Transport assay medium 100 mM Potassium Phosphate pH 6.9; Antibiotics Spectinomycin 50 ug/ml and Tetracyclin 20 ug/ml, IPTG 100 uM.

5 b. DNA techniques

c. Growth of cells : Strains constructed using recombinant methods and wild-type *P. citrea* and *K. oxytoca* were grown in either M9 medium containing DKG as the sole carbon source or MIII medium with fructose as the sole carbon source or with MIII having mixed carbon source such  
10 as fructose, Gluconate and DKG in the range from 0.1 % to 1%. Cells were grown between 20-37°C but preferably below 30°C. Cells were preferably grown at neutral pH but the range comprised of from pH 5-8. *P. citrea* cells grown in a fermenter from a seed flask used fermentation medium using either fructose or glucose feed.

15 d. DKG uptake biochemical assay: Samples of fermentation broth containing cells were withdrawn from respective growth apparatus and were quenched on ice-water bath. The fermentation broth was centrifuged and supernatant was discarded. The cell pellet was washed using 0.95 ice-cold saline solution followed by 2 washes by DKG uptake assay buffer 100 mM ice-cold Potassium phosphate pH 6.9. Cells were resuspended in the same assay  
20 buffer to an OD of 12 at 550 nm and were incubated at room temperature or preferably at 28°C. DKG uptake assay was started by mixing the cells with C-14 enriched radio-isotoped 2,5 DKG. Time course of DKG uptake was performed using vacuum/filter based quenching using ice-cold assay buffer. DKG uptake measurement were done by radioisotope incorporation in the cells and the data obtained was plotted against time to give the DKG  
25 uptake rate.

Another assay using silicone oil for cellular DKG uptake and metabolism study was also performed (Johnson, J.D. et al., J. Lab. Clin. Med., 1980, 95: 429-439). 100ul of silicon oil was added to epitubes ready for the assay. Cells and the 14C (U) enriched 2,5-DKG was mixed and then at a regular time intervals 100ul of the cell/substrate mixture was withdrawn and  
30 added to the epitubes containing silicon oil, centrifuged for 15 seconds and then immediately frozen in dryice/ethanol bath. After five minutes, tip of the epitube containing cell pellet is cut directly into a scintillation vial. Rest of the tube is recut just above the frozen portion and dropped into another vial containing scintillation fluid. Counts were measured after an overnight to facilitate cell pallet loosening from the epi tip. The difference between the loss of  
35 counts from top and appearance of counts in the pallet is due to the cell metabolism and

released CO<sub>2</sub>. At the end of the uptake assay, when cells were permeabilized, the low molecular cell contents leaked out and provided information about the accumulated imported substrate and fate of substrate further metabolized to become cellular component.

- 5 e. DKG reductases assay: Cell pellets from each fermentor were collected and frozen at -70°C for approx. 24 hours. Pellets at the 15 and 25 hour time points were thawed on ice and French pressed in 50 mM PIPES buffer, pH 6.5. The extracts were spun for 2 min. X 14 K rpm on a bench-top centrifuge, and subsequently measured for total protein concentration and reductase activity. All samples were measured by Bradford and BCA assays for protein, and  
10 diluted as needed for accurate rate assays. The assays were measured against background rates which contained all but the 2,5-DKG (and were all less than 10% of the total rates). The buffer contained: 50 mM PIPES pH 6.5, 150 uM NADPH and 5 mM 2,5-DKG.

Another assay using silicon oil for cellular DKG uptake and metabolism study was also  
15 performed (Johnson, J.D., et al., J. Lab. Clin. Med. 1980, 95:429-439). 100 ul of silicon oil was added to epitube ready for the assay. Cells and 14C (U) enriched 2,5-DKG was mixed and then at a regular time intervals (for example about every 10 seconds) 100ul of the cell/substrate mixture was withdrawn and added to the epitubes containing silicon oil, centrifuged for 15 minutes and then immediately frozen in dry ice/ethanol bath. After 5  
20 minutes, the tip of the epitube containing the cell pellet is cut directly into a scintillation vial. The rest of the tube is recut just above the frozen portion and dropped into another vial containing the scintillation fluid. Counts were measured after an overnight to facilitate cell pallet loosening from the epi tip. The difference between the loss of counts from top and appearance of counts in the pallet is due to the cell metabolism and released CO<sub>2</sub>. At the end  
25 of the uptake assay, when cells were permeabilized, the low molecular cell contents leaked out and provided information about the accumulated imported substrate and fate of substrate further metabolized to become cellular components.

#### Example II

30

Example II provides the basis that transporters of substrate may be rate limiting in a whole-cell bioconversion

This example narrates the key steps of 2KLG formation from glucose which can be  
35 compartmentalized into four parts (Fig. 5) . Production of the key intermediate 2,5-DKG using three periplasmic enzymes in *P. citrea* at 14-15 g/l/hr rate (Sonoyama, et al, Appl. Environ.

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Microbiol., 1982, 43:1064-1069). The second part is the rate by which DKG is needed to be transported in the cell's cytoplasmic space. The third is the rate of conversion of DKG to 2KLG using DKG reductases (US Patent No. 5,032,514). DKG to 2KLG conversion is not the rate limiting when DKG reductase is overexpressed. When in our current 2KLG production fermentations, inducible plasmids were used to both increase and decrease reductase specific activities relative to our typical fermentations, which presently use pD92, in which DKG reductase is under a constitutive trp promoter. The inducible plasmid, pD23, is under a taq promoter, and can be induced with IPTG. Three fermentors were run, one with pD92, and two with pD23, one of which was induced with IPTG. The control pD92 and the induced pD23 produced nearly identical levels of 2-KLG, while the uninduced pD23 made significantly less. Assays of reductase specific activities show that relative to the control pD92, the uninduced plasmid made less than half the levels of reductase, while the induced pD23 made more than twice as much. These results indicate that the level of reductase activity in our 2KLG producing *P. citrea* fermentation is not the bottleneck for the production of 2-KLG.

Strains	Protein Concentration	Reductase Activity	Specific Activity
pD92 (control)	3.2 mg/ml	25.0 u/ml	7.8 u/mg
pD23 (uninduced)	3.7 mg/ml	12.0 u/ml	3.2 u/mg
pD23 (induced)	3.9 mg/ml	73 u/ml	19 u/mg

The fourth part is the transport of 2KLG which is intracellularly made and need to be exported out. The production rate of 2KLG in the fermentation ( 2.2 g/l/hr – 2.7 g/l/hr) is considered to be equal to the export rate of 2KLG from the cell. It is argued, if the rate of export of 2KLG is limiting then cells will accumulate 2KLG in side the cell and cells will not be able to function at their metabolic potential and eventually die. However, 2KLG production cells of *P. citrea* do not exhibit either of these conditions and intracellular measurements of 2KLG remain 10-20 fold below the maximum concentration of 2KLG produced. It is thus conceived that 2KLG export is also not a rate limiting step in the production of 2KLG.

### Example III

Example III provides the proof that indeed the transport rate of substrate in to the cell for bioconversion can be the rate limiting step.

- 30 -

Cell pellets from three different times of the fermentation process, seed-flask stage, fructose feed stage and glucose feed stage were collected and processed as described in the experimental. The DKG uptake assay using these cell-types gave 0.5 g/l/hr, 2.7 g/l/hr and 2.75 g/l/hr DKG uptake rate of bring in the DKG to get converted to KLG (Fig 9). The rate of  
5 DKG import is same as KLG export. This result thus demonstrate that the rate of bioconversion of DKG to KLG is dependent upon the import rate of 2,5-DKG into the cell. Thus this invention will demonstrate that by increasing the DKG uptake transporters by overexpression will enhance the import rate of 2,5-DKG and thus will enhance the 2KLG production rate. Those expert in this art, can visualize that the key limiting factor in various  
10 biotransformations using whole cell conversion methods may indeed be the import and the import rate of substrate into the cell for the biotransformation.

#### Example IV

15 Example IV provides the discovery of a 2,5-DKG transporter in *K. oxytoca* using DKG uptake assay. WO 002170 describes the identification and sequencing of an operon from *Klebsiella oxytoca*, designated the *yia* operon, which contains eight putative open reading frames. The functions of these polypeptides encoded by the individual open reading frames in the *yia* operon are not described in WO002170. Disruption of this Operon removed the ability of *K.*  
20 *oxytoca* to use ascorbic acid as sole carbon source. It is known that ascorbic acid is an oxidatively unstable substance and it decomposes to 2,3-DKG by air oxidation (Kimaya, S., J. Vitaminol., 1961, 7:19-26). It was thus reasonable to suggest that it is 2,3-DKG which is the real substrate for growth. One of the open reading frames in the *yia* operon, designated as *yiaX2*, encoded a transporter type transmembrane protein and was thus considered a  
25 candidate for 2,3-DKG permease. In light of the parallel search of finding 2,5-DKG permease, it was thus realized that 2,3-DKG and 2,5-DKG being analogous molecules, it may be possible that *yiaX2* can transport 2,5-DKG and other sugar keto acids such as 2KLG.

In order to determine whether *yiaX2* can transport 2,5-DKG, and 2-KLG this gene was deleted  
30 from the chromosome of *K. oxytoca* strain M5a1 (see, for example, Streicher et al., Proc. Natl. Acad. Sci. 68: 1174-1177 (1971)). The *yiaX2* deletion mutant designated as MGK002 was created using standard molecular biology protocols known in the art (see for example, Hamilton et al., J. bacterial. 171:4617-4622 (1989)). Another *K. oxytoca* strain designated as Tester strain was created by adding back the plasmidly encoded and *lac* operon regulated  
35 *yiaX2* gene to *K. oxytoca* strain MGK002. DKG uptake assay under +/- IPTG induction using



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MGK002 and Tester strain confirmed that *viaX2* encoded a poly peptide having 2,5-DKG transport activities (Fig 9).

#### Example V

5

Example V provides the selection methodology for screening 2,5-DKG permeases from microorganisms

With the information that *viaX2* gene encoded a transporter protein having 2,5-DKG transport activities, it became possible to design a selection host for finding 2,5-DKG transporter protein of *P. citrea* and other biological sources. *K. oxytoca* with *viaX2* gene deletion and addition of genes to express enzymes involved in the catabolism of 2,5-DKG to gluconic acid, which is assessable to the central metabolism of an organism. Enzymes capable of catabolizing 2,5-DKG to gluconic acid are encoded by *tkr* and *idn* genes of the *tkr idnD idnO* operon present in some Gram negative organisms (Bausch, C., et al, J. Bacteriol., 1998, 180:3704-3710).

15

The resulting tester strain of *K. oxytoca* was *viaX2[tkr idn]* and had all the components needed for growth on 2,5-DKG as a sole carbon source except its inability to import DKG into the cytoplasm. Therefore, a nucleic acid molecule that encodes a 2,5-DKG permease, upon expression in the tester strain, should confer the ability of the tester strain to grow on 2,5-DKG. This selection methodology is shown in figure 9.

20

The cloning vector used for constructing the *P. citrea* genomic libraries is plasmid pCI1920 (Lerner et al., Nucleic Acid Res., 1994, 18:4621), a low-copy number expression vector which carries a spectinomycin/streptomycin resistance determinant. Expression is driven by the lacPO promoter/operator region which is repressed by lacIq gene product when provided by the host. Genomic DNA from *P. citrea* (ATCC 39140) was isolated using standard protocol and genomic library was created (Sambrook, et al, Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory, New York (1992)). The amplified libraries were stored in the form of Plasmid DNA for further use to find 2,5-DKG permease of *P. citrea*.

30

#### Example VI

Example VI provides the proof that by overexpressing DKG transporter in the host cell, one can enhance the DKG import rate into the cell.

35

Genomic library was introduced into tester strain *K. oxytoca* yiaX2[tkr idno] strain. Clones that grew on 2,5-DKG using M9-agar plates with 2.5% 2,5-DKG and 0.1 mM IPTG were tested for DKG uptake using radiolabeled <sup>14</sup>C (U) DKG. Various clones were found to have improved DKG uptake than the control tester strain (Fig. 9 ) Genomic library DNA from these positive clones was transformed into *P. citrea* (139-2A) and DKG uptake assay was performed to measure the improvement in DKG uptake over the *P. citrea* 139-2A strain. Three to five fold improvement in DKG uptake rate was seen in the transformants having additional copies of plasmid encoded DKG permeases found through genomic library screening and selection methodology (Fig 10).

#### Example VII

Example VII provides the proof that by overexpressing the DKG transporter in the host cell one is able to improve the production of 2KLG.

Nucleic acid molecule encoding *Pantoea citrea* DKG permease PermA (seq ID no2) when subcloned using low copy vector pBCL1920 into a *Pantoea citrea* strain 139-2A suitable for biosynthesis of 2KLG from glucose (US Patent 5,032,514), the production rate of 2KLG improved from 2.5 g/l/hr to 3.2 g/l/hr and yield on the sugar improved from 45 % to 53% (fig 11 )

#### Example VIII

Example VIII describes the characteristics of 2,5-DKG permease PermA from *P. citrea*.

This example describes the membrane topology of PermA of *P. citrea*. PFAM analysis (Hirokawa, T., et al., Bioinformatics, 1998, 4(4): 378) predicts that the PermA has 11 transmembrane spanning domains, with 8 primary domains and 3 secondary spanning domains (Fig. 12). The amino terminal is in the periplasm and carboxy terminal being localized in the cytoplasm. Two major and two minor loops exist and both periplasm and cytoplasm have one major and one minor loop. The PermA is a membrane protein with hydrophobicity of 0.62 and has molecular weight of 48 Dalton. It is a one H<sup>+</sup> associated symporter of 2,5-DKG based on the accumulation ratio analysis (Lolkema, J.S., et al. 1996, Handbook of Biological Physics, Chapter 11, 229-260). It belongs to ACS family of MFS based on the consensus sequence analysis (Pao, S.S., et al., Microbiol. Molecular Biol. Rev., 1998, 62:1-34) (Fig. 8a).

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Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced  
5 herein are hereby incorporated in their entirety.

## CLAIMS

1. A method for enhancing a host cell's biosynthetic production of 2-KLG, the method comprising:
  - a) selecting a host cell that cytosolically converts 2,5-DKG to 2-KLG;
  - b) increasing the transport of said 2,5-DKG into said host cell while maintaining the integrity of the host cell;
  - c) culturing the host cell to produce said 2-KLG; and
  - d) producing the 2-KLG.
2. The method of claim 1, wherein said increasing the transport of 2,5-DKG into said host cell includes the step of transforming into said host cell DNA encoding for one or more proteins transporting said 2,5-DKG into said host cell's cytosolic material.
3. The method of claim 2, wherein said one or more protein is selected from the group consisting of YiaX2, PE1, PE6, prmA and prmB.
4. The method of claim 2, wherein said DNA encoding for one or more proteins is expressed from genomes selected from the group consisting of YiaX2, PE1, PE6, prmA and prmB.
5. The method of claim 2, wherein said proteins is capable of [stringent] hybridization with SEQ ID NO: [consensus sequence].
6. The method of claim 2, wherein said proteins has at least 50% homology with SEQ ID NO: [consensus permease sequence].
7. The method of claim 2, wherein said proteins has at least 90% homology with SEQ ID NO: [consensus permease sequence].
8. The method of claim 2, wherein said proteins has at least 50% homology with SEQ ID NO: [whole permease sequence].
9. The method of claim 2, wherein said proteins has at least 90% homology with SEQ ID NO: [whole permease sequence].

10. The method of claim 2, wherein said proteins comprises a sequence comprising 31 residues, said residues comprising a glycine residue which corresponds to glycine 119 of PermA

11. The method of claim 11, wherein said proteins further comprises a tryptophan residue (W) which corresponds to position 136 of PermA.

12. The method of claim 11, wherein said proteins further comprises at least one amino acid residue selected from the group consisting of a phenylalanine at a position which corresponds to G138 of PermA, a glutamic acid (E) at a position which corresponds to E141 of PermA, and an arginine (R) at a position which corresponds to R142 of PermA.

14. The method for enhancing the transport of 2,5 DKG into the cytosol across the inner cell membrane, the method comprising:

Selecting a host cell; and

Transforming into said host cell DNA encoding for one or more proteins transporting of 2,5 DKG into said host cell.

15. The method of claim 14, wherein said host cell is selected from the group consisting of bacteria and yeast.

16. The method of claim 15 wherein said host cell is selected from the group consisting of *E. coli*, *pantoia*, and *klesiella*.

17. A method of overexpressing a 2,5-DKG transporter comprising the steps:

Selecting a host cell; and

Transforming into said host cell, DNA encoding for one or more 2,5-DKG transporters.

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SEQ. ID.1. *Klebsiella Oxytoca* Permease ViaX2.

atg aat ata acc tct aac tct aca acc aaa gat ata ccg cgc  
 Met Asn Ile Thr Ser Asn Ser Thr Thr Lys Asp Ile Pro Arg  
  
 cag cgc tgg tta aga atc att ccg cct ata ctg atc act tgt att att  
 Gln Arg Trp Leu Arg Ile Ile Pro Pro Ile Leu Ile Thr Cys Ile Ile  
  
 tct tat atg gac cgg gtc aat att gcc ttt gcg atg ccc gga ggt atg  
 Ser Tyr Met Asp Arg Val Asn Ile Ala Phe Ala Met Pro Gly Gly Met  
  
 gat gcc gac tta ggt att tcc gcc acc atg gcg ggg ctg gcg ggc ggt  
 Asp Ala Asp Leu Gly Ile Ser Ala Thr Met Ala Gly Leu Ala Gly Gly  
  
 att ttc ttt atc ggt tat cta ttt tta cag gtt ccc ggc ggg aaa att  
 Ile Phe Phe Ile Gly Tyr Leu Phe Leu Gln Val Pro Gly Gly Lys Ile  
  
 gcc gtt cac ggt agc ggt aag aaa ttt atc ggc tgg tcg ctg gtc gcc  
 Ala Val His Gly Ser Gly Lys Lys Phe Ile Gly Trp Ser Leu Val Ala  
  
 tgg gcg gtc atc tcc gtg ctg acg ggg tta att acc aat cag tac cag  
 Trp Ala Val Ile Ser Val Leu Thr Gly Leu Ile Thr Asn Gln Tyr Gln  
  
 ctg ctg gcc ctg cgc ttc tta ctg ggc gtg gcg gaa ggc ggt atg ctg  
 Leu Leu Ala Leu Arg Phe Leu Leu Gly Val Ala Glu Gly Gly Met Leu  
  
 ccg gtc gtt ctc acg atg atc agt aac tgg ttc ccc gac gct gaa cgc  
 Pro Val Val Leu Thr Met Ile Ser Asn Trp Phe Pro Asp Ala Glu Arg  
  
 ggt cgc gcc aac gcg att gtc att atg ttt gtg ccg att gcc ggg att  
 Gly Arg Ala Asn Ala Ile Val Ile Met Phe Val Pro Ile Ala Gly Ile  
  
 atc acc gcc cca ctc tca ggc tgg att atc acg gtt ctc gac tgg cgc  
 Ile Thr Ala Pro Leu Ser Gly Trp Ile Ile Thr Val Leu Asp Trp Arg  
  
 tgg ctg ttt att atc gaa ggt ttg ctc tcg ctg gtt gtt ctg gtt ctg  
 Trp Leu Phe Ile Ile Glu Gly Leu Leu Ser Leu Val Val Leu Val Leu  
  
 tgg gca tac acc atc tat gac cgt ccg cag gaa gcg cgc tgg att tcc  
 Trp Ala Tyr Thr Ile Tyr Asp Arg Pro Gln Glu Ala Arg Trp Ile Ser  
  
 gaa gca gag aag cgc tat ctg gtc gag acg ctg gcc gcg gag caa aaa  
 Glu Ala Glu Lys Arg Tyr Leu Val Glu Thr Leu Ala Ala Glu Gln Lys  
  
 gcc att gcc ggc acc gag gtg aaa aac gcc tct ctg agc gcc gtt ctc  
 Ala Ile Ala Gly Thr Glu Val Lys Asn Ala Ser Leu Ser Ala Val Leu  
  
 tcc gac aaa acc atg tgg cag ctt atc gcc ctg aac ttc ttc tac cag  
 Ser Asp Lys Thr Met Trp Gln Leu Ile Ala Leu Asn Phe Phe Tyr Gln  
  
 acc ggc att tac ggc tac acc ctg tgg cta ccc acc att ctg aaa gaa  
 Thr Gly Ile Tyr Gly Tyr Thr Leu Trp Leu Pro Thr Ile Leu Lys Glu  
  
 ttg acc cat agc agc atg ggg cag gtc ggc atg ctt gcc att ctg ccg  
 Leu Thr His Ser Ser Met Gly Gln Val Gly Met Leu Ala Ile Leu Pro

FIG. 1A

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tac gtc ggc gcc att gct ggg atg ttc ctg ttt tcc tcc ctt tca gac  
Tyr Val Gly Ala Ile Ala Gly Met Phe Leu Phe Ser Ser Leu Ser Asp

cga acc ggt aaa cgc aag ctg ttc gtc tgc ctg ccg ctg att ggc ttc  
Arg Thr Gly Lys Arg Lys Leu Phe Val Cys Leu Pro Leu Ile Gly Phe

gct ctg tgc atg ttc ctg tcg gtg gcg ctg aaa aac caa att tgg ctc  
Ala Leu Cys Met Phe Leu Ser Val Ala Leu Lys Asn Gln Ile Trp Leu

tcc tat gcc gcg ctg gtc ggc tgc gga ttc ttc ctg caa tcg gcg gct  
Ser Tyr Ala Ala Leu Val Gly Cys Gly Phe Phe Leu Gln Ser Ala Ala

ggc gtg ttc tgg acc atc ccg gca cgt ctg ttc agc gcg gaa atg gcg  
Gly Val Phe Trp Thr Ile Pro Ala Arg Leu Phe Ser Ala Glu Met Ala

ggc ggc gcg cgc ggg gtt atc aac gcg ctt ggc aac ctc ggc gga ttt  
Gly Gly Ala Arg Gly Val Ile Asn Ala Leu Gly Asn Leu Gly Gly Phe

tgt ggc cct tat gcg gtc ggg gtg ctg atc acg ttg tac agc aaa gac  
Cys Gly Pro Tyr Ala Val Gly Val Leu Ile Thr Leu Tyr Ser Lys Asp

gct ggc gtc tat tgc ctg gcg atc tcc ctg gcg ctg gcc gcg ctg atg  
Ala Gly Val Tyr Cys Leu Ala Ile Ser Leu Ala Leu Ala Ala Leu Met

gcg ctg ctg ctg ccg gcg aaa tgc gat gcc ggt gct gcg ccg gta aag  
Ala Leu Leu Leu Pro Ala Lys Cys Asp Ala Gly Ala Ala Pro Val Lys

acg ata aat cca cat aaa cgc act gcg  
Thr Ile Asn Pro His Lys Arg Thr Ala

**FIG. 1B**

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## SEQ. ID.2. Environm ntal P rmease PE1.

atg aac agc tct acc aat gca  
Met Asn Ser Ser Thr Asn Ala

acg aaa cgc tgg tgg tac atc atg cct atc gtg ttt atc acg tat agc  
Thr Lys Arg Trp Trp Tyr Ile Met Pro Ile Val Phe Ile Thr Tyr Ser

ctg gcg tat ctc gac cgc gca aac ttc agc ttt gct tcg gca gcg ggc  
Leu Ala Tyr Leu Asp Arg Ala Asn Phe Ser Phe Ala Ser Ala Ala Gly

att acg gaa gat tta ggc att acc aaa ggc atc tcg tcg ctt ctt ggc  
Ile Thr Glu Asp Leu Gly Ile Thr Lys Gly Ile Ser Ser Leu Leu Gly

gca ctt ttc ttc ctc ggc tat ttc ttc ttc cag atc ccg ggg gcg att  
Ala Leu Phe Phe Leu Gly Tyr Phe Phe Phe Gln Ile Pro Gly Ala Ile

tac gcg gaa cgc cgt agc gta cgg aag ctg att ttc atc tgt ctg atc  
Tyr Ala Glu Arg Arg Ser Val Arg Lys Leu Ile Phe Ile Cys Leu Ile

ctg tgg ggc gcc tgc gcc tcg ctt gac cgg gat ggt gca caa tat tcc  
Leu Trp Gly Ala Cys Ala Ser Leu Asp Arg Asp Gly Ala Gln Tyr Ser

agc gct ggc tgg cga tcc gtt tta ttc tcg gct gtc gtg gaa gcg gcg  
Ser Ala Gly Trp Arg Ser Val Leu Phe Ser Ala Val Val Glu Ala Ala

gtc atg ccg gcg atg ctg att tac atc agt aac tgg ttt acc aaa tca  
Val Met Pro Ala Met Leu Ile Tyr Ile Ser Asn Trp Phe Thr Lys Ser

gaa cgt tca cgc gcc aac acc ttc tta atc ctc ggc aac ccg gtc acg  
Glu Arg Ser Arg Ala Asn Thr Phe Leu Ile Leu Gly Asn Pro Val Thr

gta ctg tgg atg tcg gtg gtc tcc ggc tac ctg att cag tcc ttc ggc  
Val Leu Trp Met Ser Val Val Ser Gly Tyr Leu Ile Gln Ser Phe Gly

tgg cgt gaa atg ttt att att gaa ggc gtt ccg gcc gtc ctc tgg gcc  
Trp Arg Glu Met Phe Ile Ile Glu Gly Val Pro Ala Val Leu Trp Ala

ttc tgc tgg tgg gtg ctg gtc aaa gtt aaa ccg tcg cag gtg aac tgg  
Phe Cys Trp Trp Val Leu Val Lys Val Lys Pro Ser Gln Val Asn Trp

ttg tcg gaa aac gag aaa gcc gcg ctg cag gcg cag ctg gag agc gag  
Leu Ser Glu Asn Glu Lys Ala Ala Leu Gln Ala Gln Leu Glu Ser Glu

cag cag ggt att aaa gcc gtg cgt aac tac ggc gaa gcc ttc cgc tca  
Gln Gln Gly Ile Lys Ala Val Arg Asn Tyr Gly Glu Ala Phe Arg Ser

cgc aac gtc att cta ctg tgc atg cag tat ttt gcc tgg agt atc ggc  
Arg Asn Val Ile Leu Leu Cys Met Gln Tyr Phe Ala Trp Ser Ile Gly

gtg tac ggt ttt gtg ctg tgg ttg ccg tca att att cgc agc ggc ggc  
Val Tyr Gly Phe Val Leu Trp Leu Pro Ser Ile Ile Arg Ser Gly Gly

gtc aat atg ggg atg gtg gaa gtc gcc tgg ctc tct tcg gtg cct tat  
Val Asn Met Gly Met Val Glu Val Gly Trp Leu Ser Ser Val Pro Tyr

FIG. 1C

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ctg gcc gcg act att gcg atg atc gtc gtc tcc tgg gct tcc gat aaa  
Leu Ala Ala Thr Ile Ala Met Ile Val Val Ser Trp Ala Ser Asp Lys

atg cag aac cgt aaa ctg ttc gtc tgg ccg ctg ctg ctg att ggc gga  
Met Gln Asn Arg Lys Leu Phe Val Trp Pro Leu Leu Leu Ile Gly Gly

ctg gct ttt att ggc tca tgg gcc gtc ggc gct aac cat ttc tgg gcc  
Leu Ala Phe Ile Gly Ser Trp Ala Val Gly Ala Asn His Phe Trp Ala

tct tat acc ctg ctg gtg att gcc aat gcg gca atg tac gcc cct tac  
Ser Tyr Thr Leu Leu Val Ile Ala Asn Ala Ala Met Tyr Ala Pro Tyr

ggt ccg ttt ttc gcc atc att ccg gaa atg ctg ccg cgt aac gtc gcc  
Gly Pro Phe Phe Ala Ile Ile Pro Glu Met Leu Pro Arg Asn Val Ala

ggt ggc gca atg gcg ctc atc aac agc atg ggg gcc tta ggt tca ttc  
Gly Gly Ala Met Ala Leu Ile Asn Ser Met Gly Ala Leu Gly Ser Phe

ttt ggt tcg tgg ttc gtg ggc tac ctg aac ggc acc acc ggc agt cca  
Phe Gly Ser Trp Phe Val Gly Tyr Leu Asn Gly Thr Thr Gly Ser Pro

tca gcc tca tac att ttc atg gga gtg gcg ctt ttc gcc tcg gta tgg  
Ser Ala Ser Tyr Ile Phe Met Gly Val Ala Leu Phe Ala Ser Val Trp

ctt act tta att gtt aag cct gct aac aat caa aag ctc ccc atc ggc  
Leu Thr Leu Ile Val Lys Pro Ala Asn Asn Gln Lys Leu Pro Ile Gly

gct cgt cac gcc  
Ala Arg His Ala

**FIG. 1D**

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## SEQ. ID.3. Envir nmental Permeas PE6.

```

                                atg aat aca gcc tct gtt tct
                                Met Asn Thr Ala Ser Val Ser

gtc acc caa agc cag gcg atc ccc aaa tta cgc tgg ttg aga ata gtg
Val Thr Gln Ser Gln Ala Ile Pro Lys Leu Arg Trp Leu Arg Ile Val

ccg cct att ctt att acc tgc att att tcc tat atg gac cgg gtg aac
Pro Pro Ile Leu Ile Thr Cys Ile Ile Ser Tyr Met Asp Arg Val Asn

atc gcc ttc gcc atg ccc ggc ggc atg gac gat gaa ctg ggc atc acc
Ile Ala Phe Ala Met Pro Gly Gly Met Asp Asp Glu Leu Gly Ile Thr

gcc tcg atg gcc ggg ttg gcc ggc ggt att ttc ttt atc ggt tat ctg
Ala Ser Met Ala Gly Leu Ala Gly Gly Ile Phe Phe Ile Gly Tyr Leu

ttc ttg cag gta ccc ggc ggc aag ctg gcg gtg tac ggc aac ggc aag
Phe Leu Gln Val Pro Gly Gly Lys Leu Ala Val Tyr Gly Asn Gly Lys

aaa ttc atc ggt tgg tcg ttg ttg gcc tgg gcg gtg att tcc gtg ctg
Lys Phe Ile Gly Trp Ser Leu Leu Ala Trp Ala Val Ile Ser Val Leu

acc ggg ctg gtc acg aat cag tat caa ttg ctg ttc ctg cgc ttc gcc
Thr Gly Leu Val Thr Asn Gln Tyr Gln Leu Leu Phe Leu Arg Phe Ala

ctc ggc cgt ttc cga agc ggc atg ctg cgg tgg gtg ctg acc atg atc
Leu Gly Arg Phe Arg Ser Gly Met Leu Arg Trp Val Leu Thr Met Ile

agc aac tgg ttc ccg gac aag gaa cgc ggg cgc gcc aac gcc atc gtc
Ser Asn Trp Phe Pro Asp Lys Glu Arg Gly Arg Ala Asn Ala Ile Val

atc atg ttc gtg ccg atc gcc ggc atc ctt acc gca ccg ctg tcc ggc
Ile Met Phe Val Pro Ile Ala Gly Ile Leu Thr Ala Pro Leu Ser Gly

tgg atc atc acc gcc tgg gac tgg cgc atg ctg ttc ctg gtc gag ggc
Trp Ile Ile Thr Ala Trp Asp Trp Arg Met Leu Phe Leu Val Glu Gly

gcg ctg tcg ctg gtc gtg atg gtg ctg tgg tat ttc acc atc agc aac
Ala Leu Ser Leu Val Val Met Val Leu Trp Tyr Phe Thr Ile Ser Asn

cgt cca caa gag gcc aaa agg att tcg cag gcg gaa aaa gat tat ctg
Arg Pro Gln Glu Ala Lys Arg Ile Ser Gln Ala Glu Lys Asp Tyr Leu

atc aaa acg ctg cac gac gaa cag ttg ctg atc aaa ggc aaa acg gtg
Ile Lys Thr Leu His Asp Glu Gln Leu Leu Ile Lys Gly Lys Thr Val

cgc aac gcc tcg ctg cgt cgg gtg ctg ggc gac aaa atc atg tgg aag
Arg Asn Ala Ser Leu Arg Arg Val Leu Gly Asp Lys Ile Met Trp Lys

ttc ttc tac cag acc ggg ata tac ggc tac acc ctg tgg ctg ccg acc
Phe Phe Tyr Gln Thr Gly Ile Tyr Gly Tyr Thr Leu Trp Leu Pro Thr

att ctc aag ggg ctc acc aac ggc aat atg gag cag gtc ggg atg ctg
Ile Leu Lys Gly Leu Thr Asn Gly Asn Met Glu Gln Val Gly Met Leu

```

**FIG. 1E**

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gct atc ctg ccc tat atc ggc gcc atc ttc ggc atg ctg atc att tcc  
Ala Ile Leu Pro Tyr Ile Gly Ala Ile Phe Gly Met Leu Ile Ile Ser

acc ctc tcc gat cgc acc ggc aag cgc aaa gtg ttc gtc gca ctg ccg  
Thr Leu Ser Asp Arg Thr Gly Lys Arg Lys Val Phe Val Ala Leu Pro

ctg gcc tgc ttt gcc atc tgc atg gcg ctg tcg gtg ctg ctg aag gat  
Leu Ala Cys Phe Ala Ile Cys Met Ala Leu Ser Val Leu Leu Lys Asp

cac atc tgg tgg tcg tac gcg gcg ctg gtg ggc tgt ggc gtc ttt acc  
His Ile Trp Trp Ser Tyr Ala Ala Leu Val Gly Cys Gly Val Phe Thr

cag gcc gcc gcc ggg gtg ttc tgg acc att ccg ccc aag ctg ttt aac  
Gln Ala Ala Ala Gly Val Phe Trp Thr Ile Pro Pro Lys Leu Phe Asn

gcc gaa atg gcc ggc ggc gcg cgc ggc gtg atc aat gca ctg ggc aac  
Ala Glu Met Ala Gly Gly Ala Arg Gly Val Ile Asn Ala Leu Gly Asn

ctc ggc ggt ttc tgc ggc ccc tat atg gtc ggc gtg ttg atc acc ttg  
Leu Gly Gly Phe Cys Gly Pro Tyr Met Val Gly Val Leu Ile Thr Leu

ttc agc aaa gac gtc ggc gtt tac agc ctc gcg gtg tcg ctg gcc tcc  
Phe Ser Lys Asp Val Gly Val Tyr Ser Leu Ala Val Ser Leu Ala Ser

gcc tcg gtg ctg gcg ttg atg ctg ccg aac aga tgc gac caa aaa gcg  
Ala Ser Val Leu Ala Leu Met Leu Pro Asn Arg Cys Asp Gln Lys Ala

ggg gcc gaa  
Gly Ala Glu

**FIG. 1F**

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**SEQ. ID.4. *Pantoea Citrea* Permease A.****DNA seq.**

ATGCAAAAATCACAGCCGGGAACCCGCTGGTTTCGGATTATTGTGCCGATC  
CTGATAGCCTGCATCATGTCGTTTATGGATCGGGTAAATATCAGTTTCGCAT  
TGCCGGGCGGTATGGAGCAGGATCTGCTGATGTCCAGCCAGATGGCCGGG  
GTAGTTAGCGGTATTTTCTTTATTGGTTATCTGTTTTTGCAGGTTCTGGTGG  
GCATATCGCAGTACGTGGCAGTGGTAAACGTTTTATTGCCTGGTCGCTTGT  
GCCTGGGCGCGTTGTTTCTGTCGCTACCGGGTTTGTGACTCATCAGTACCAG  
CTGTTGATTTTACGTTTTGCACTGGGGGTCTCTGAAGGTGGGATGTTGCCG  
GTAGTTCTGACAATGGTCAGCAACTGGTTTCCTGAAAAAGAGCTGGGGCGT  
GCTAATGCATTTGTCATGATGTTCCGCCCCGCTTGGCGGAATGATTACCGCC  
CCTGTCTCCGGATGGATTATTGCACTGCTAGACTGGCGCTGGTTATTTATTA  
TCGAAGGATTACTGTCGGTAGTGGTTCTGGCAGTCTGGTGGCTGATGGTCA  
GTGACCGCCCTGAAGATGCCCGTTGGCTGCCGGCAGCAGAACGGGAATAT  
CTGCTGCGCGAAATGGCCCGTGACAAGGCCGAGCGGAGCAAACCTCCCTCC  
GATCAGTCATGCTCCCCTGCAAGAGGTTTTCCATAACCCGGGCCTGATGAA  
GTTAGTGATTCTGAACTTTTCTATCAGACAGGTGATTACGGATAGACTCTGT  
GGCTGCCGACTATTATCAAAAACCTGACCGGAGCTAGTATTGGTAACGTCG  
GTTTGCTGACAGTGCTACCTTTTATCGCGACGTTATCAGGGATTTATGTCGT  
CTCTTACCTGAGCGATAAAACCGGCCAAACGTCGGCAATGGGTGATGATTTT  
TCTGTTCTGTTTTGCGGCCTGCCTGTTGGCCTCAGTCTGTTACGTGAATTT  
GTGCTGGCTGCTTATCTGGCTCTGGTGGCTTGC GGCTTTTTCTGAAAGCA  
GCCACCAGCCCGTTCTGGAGTATCCGGGACGTATTGCACCGCCGGAAGC  
AGCCGGTAGTGCCCGTGGTGTAATTAACGGACTGGGGAATCTGGGCGGTTT  
CTGCGGCCCCCTGGCTGGTCGGATTAATGATCTACCTGTACGGACAGAATGC  
AGCCGTTGTTACTCTGGCAGGCTCTCTGATCATTGCCGGGATTATTGCGGC  
ATTACTGCCAACGCAGTGTGATCTGCGCCCGGCAGAGGCACGGCAGCAGA  
ATTTACCCACGTATTCATGATGCCAAA

**Protein seq.**

MQKSQPGTRWFRIVPILACIMSFMDRVNISFALPGGMEQDLLMSSQMAGVVS  
GIFFIGYLFLQVPGGHIAVRGSGKRFIASLVAWAVVSVATGFVTHQYQLLILRF  
ALGVSEGGMLPVVLTMVSNWFPEKELGRANAFVMMFAPLGGMITAPVSGWIIA  
LLDWRWLFIEGLLSVVVLAVWWLMVSDRPEDARWLPAAREYLLREMARDKA  
ERSKLPPISHAPLQEVFHNPLMKLVILNFFYQTGDYGYTLWLPTIKNLTGASIG  
NVGLLTVLPFIATLSGIYVVSYSCLKTKRRQWVMISLFCFAACLLASVLLREFVL  
AAYLALVACGFFLKAATSPFWSIPGRIAPPEAAGSARGVINGLGNLGGFCGPWL  
VGLMIYLYGQNAAVVTLAGSLIAGIIAALLPTQCDLRPAEARQQNFTPRIHDAK

**FIG. 1G**

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**SEQ. ID.5. *Pantoea Citrea* Permease B.****DNA seq.**

ATGCCGGTGATTTTTATTACTTACAGCCTGGCATATCTGGATCGGGCCAACT  
ACGGCTTTGCTGCTGCCTCTGGGATTGAAGCAGATCTTGGAATTAGCCGTG  
GCACCTCCTCTCTGATTGGAGCACTGTTCTTTCTCGGCTACTTCATTTTTCA  
GGTGCCCGGGGCAATTTATGCAGTGAAACGCAGTGTCCGTAAACTGGTGTT  
TACCAGCCTGCTGTTGTGGGGATTTGTGCCGCTGCGACCGGACTTATCAG  
CAATATTCCGGCTCTGATGGTGATCCGCTTTGTTCTGGGTGTTGTTGAAGCC  
GCAGTGATGCCAGCGATGCTGATTTACATCAGCAACTGGTTCACCCGTGAG  
GAACGTTACGGGGCTAATACCTTTCTGGTATTAGGTAACCCGGTCACGGTG  
TTATGGATGTCTATTGTTTCCGGATATCTGATCAATGCTTTTGGCTGGCGGG  
AAATGTTTATTTTCGAGGGTGTGCCTGCCTTAATCTGGGCCATCTTCTGGTG  
GTTTATTGTCCGGGACAAACCGGAGCAGGTGAGCTGGCTGACAGAAACAGA  
AAAGCAGCAACTGGCCAGTGCAATGGCTGAAGAGCAGCAGGCAATACCAC  
CGATGCGCAATGTGCCGCAGGCCCTGCGTTCCCGCAATGTGGTGGTACTG  
TGCCTGTTACACGCTCTGTGGAGCATCGGAGTGTATGGTTTTATGATGTGGA  
TGCCATCGATACTGCGTAGCGCTGCATCAATGGACATTGTCCGGGTAGGCT  
GGCTGGCCGCAGTTCCGTATCTGGCCGCGATTATTACTATGCTGGTGATT  
CATGGCTGTCAGATAAAACCGGGCTGCGTCGGCTTTTTATCTGGCCATTATT  
GCTGATTGCGTCAGTTACTTTTTTTGGGTCCTGGTTACTTGGGAGCTACTCA  
TTCTGGTTTTCTATGGCTTGCTGGTACTGGCTGCTGCTTGATGTATGCC  
CGTATGGACCGTTTTTTGCGTTGATTCTGAATTGCTGCCAAAAATGTGGC  
GGGATTTCTATCGGGTTAATTAAGTGTGCGGGGCGCTGGGAGCTTTTGC  
CGGAGCCTGGCTGGTGGGCTATCTTAATGGTCTGACCGGTGGTCCGGGGG  
CTTCTTACACTTTTATGGCCATTGCATTGCTGGTTTCTGTAGGGTTGGTGGT  
TTCCTGAAAGTCCCTTCAGGGAATTTGGTCACTCGTCGGTTGCTGAAAGGT  
GATGCAAAG

**Protein seq.**

MPVIFITYSLAYLDRANYGFAAASGIEADLGISRGTSLLIGALFFLG YFIFQVPGAI  
YAVKRSVRKLVFTSLLLWGFCAAATGLISNIPALMVIRFVLGVVEAAMPAMLIYI  
SNWFTRQERSRANTFLVLGNPVTVLWMSIVSGYLINAFGWREMFIFEGVPALIW  
AIFWWFIVRDKPEQVSWLTETEKQQLASAMAEQQAIPPMRNVPOALRSRNVV  
VLCLLHALWSIGVYGFMWMPMSILRSAASMDIVRVGWLAAPYLAAILTMLVISW  
LSDKTGLRRLFIWPLLLIASVTFFGSWLLGSYSFWFSYGLLVLAACMYAPYGP  
FFALIPPELLPKNVAGISIGLINCCGALGAFAGAWLVGYLNGLTGGPGASYTFMAI  
ALLVSVGLVFFLKVPSGNLVTRRLLKGDAK

**FIG. 1H**

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**SEQ. ID.6. *Pantoea Citrea* P rmease YlaX2.****DNA seq.**

ATGAATACTCATCAGGCAGCCAAAGGTATTGCTATACCTAAACAACGGTGGC  
TGAGAATTATCTCGCCAATAATTATCACCTGCATTATTTCTTATATGGACCGG  
GTCAATATTGCTTTTGCCATGCCTGGAGGAATGGATAAAGATTTATCCGTCT  
CTGCCAGTATGGCCGGATTGGCCGGCGGAATATTTTTATAGGTTATCTGTT  
CTTACAGGTTCCCGGTGGAAAAATAGCCGTACACGGTAGTGGTAAGAAATT  
TATTGGCTGGTCACTGGTGGCCTGGGCAGTAATTTCAAGTTCTGACCGGCAT  
GGTTACTAACCAGTATCAGTTGCTGTTTTACGCTTTCTTCTGGGGGTATCA  
GAAGGGGGTATGTTGCCTGTTGTTCTCACTATCATCAGTAACTGGTTTCCAG  
ATCGTGAACGCGGCAGAGCCAATCAATCGTGATTATGTTTGTACCCATCG  
CCGGAATAATCTCCGCCCCGCTGGCAGGCTGGTTAATCTCTTCTCTGGACT  
GGCGCTGGCTGTTTTATATTGAAGGTTTACTTTCTCTGGCGGTACTGCTGCT  
GTGGGCACTGACTATTTCTGACCGCCCCGCCGAAGCACGCTGGATCTCCC  
GGGCCGAAAAAGATTATCTGCTCAAACCTTGCGGGAAGAGCAGATAGCTC  
ACCAACCCCTTTGCGTAAAGTCACTCTGTCGTCAGTGCTGGAAAACAGAA  
CTTTATGGCTGCTGATTGCACTTAATTTTTCTATCAGGCCGGAATCTATGGC  
TACACATTGTGGCTGCCGACCATTTTAAAAGATATGGCGCATAGCAGCATGT  
CCCTGGTCGGTTTACTGGCAATTCTGCCTTATGTCGCCGCCATGGCCGGTA  
TGTTCTGTTTTCCCGGTTATCGGATAAGAGTGGCAAACGGCGCCGTTATGT  
CATTCTGCCCTTGTTCCGATTCCGCTTATGCATGGCATTGTCAGTAATCAGC  
CAGGGCCATTTATGGATTCTTACAGCGCACTGATTGGCTGTGGTTTTTTCC  
TGCAGTCAGCTGCTGGGGTGTTTTGGGCAATTCCGGCCCCGGTTGTTTAGTG  
CCGAGGTCGCAGGCAGTGCCCGTGGGGTAATTAATGCCTTAGGTAATCTTG  
GCGGTTTTTGTTGGCCCCTATCTGGTCGGCATCTTTATTACTTTTTATAGCCA  
GGCGGCCGGTGTCTATTTCTGGCAATTTGCTGGCGATTGCCGGAGCTCT  
GGCATTTTGCCTGCCACGCCGCTGCGATATGTCAGCCAGCGAAATCGCCGC  
TGAAGATGCAAAGATTGAACCGCTGCCCGGACATGCCGGGAGGGTATTA

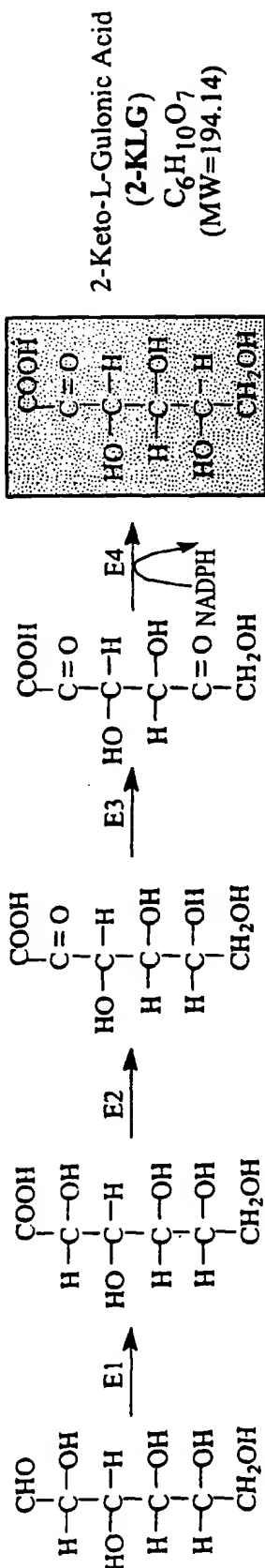
**Protein seq.**

MNTHQAAKGIAIPKQRWLRISPIITCIISYMDRVNIAFAMPGGMDKDLVSASMA  
GLAGGIFFIGYLFLQVPGGKIAVHSGKKFIGWSLVAVAVISVLTGMVTNQYQLL  
FLRFLLVSEGGMLPVVLTIIISNWFPDRERGRANSIVIMFVPIAGIISAPLAGWLIS  
SLDWRWLFYIEGLLSLAVLLLWALTISDRPAEARWISRAEKDYLLKTLREEQIAH  
QPPLRKVTLSSVLENRTLWLLIALNFFYQAGIYGYTLWLPTILKDMAHSSMSLVG  
LLAILPYVAAMAGMFLFSRLSDKSGKRRRYVILPLFGFALCMALSVISQGHLLWIS  
YSALIGCGFFLQSAAGVFWAIPARLFSAEVAGSARGVINALGNLGGFCGPYLVG  
IFITFYSQAAGVYFLAISLAIALGALAFCLPRRCDSASEIAAEDAKIEPLPGHAGR  
VL

**FIG. 11**

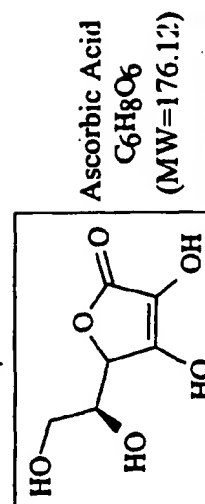
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**Integrated Schematics Glucose to Ascorbic Acid**



2-KLG Recovery

Thermal acid-catalyzed  
cyclization, separation,  
and crystallization



**Biocatalytic Conversion of Glucose  
to 2-Keto-L-Gulonic Acid**

E1 - glucose dehydrogenase  
E2 - gluconic acid dehydrogenase  
E3 - 2-keto-D-gluconic acid dehydrogenase  
E4 - 2,5-diketo-D-gluconic acid reductase

**FIG..2**

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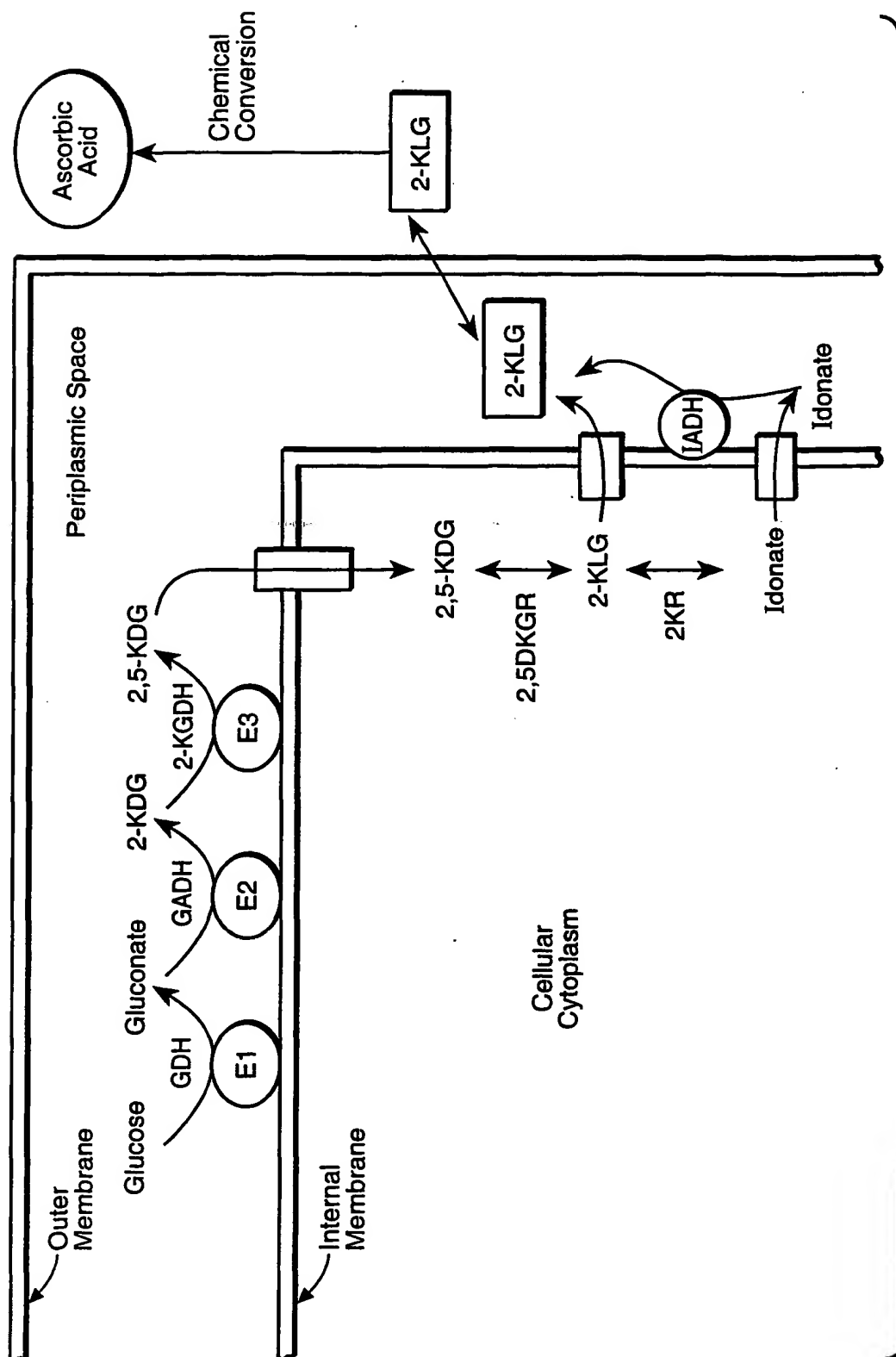


FIG. 3



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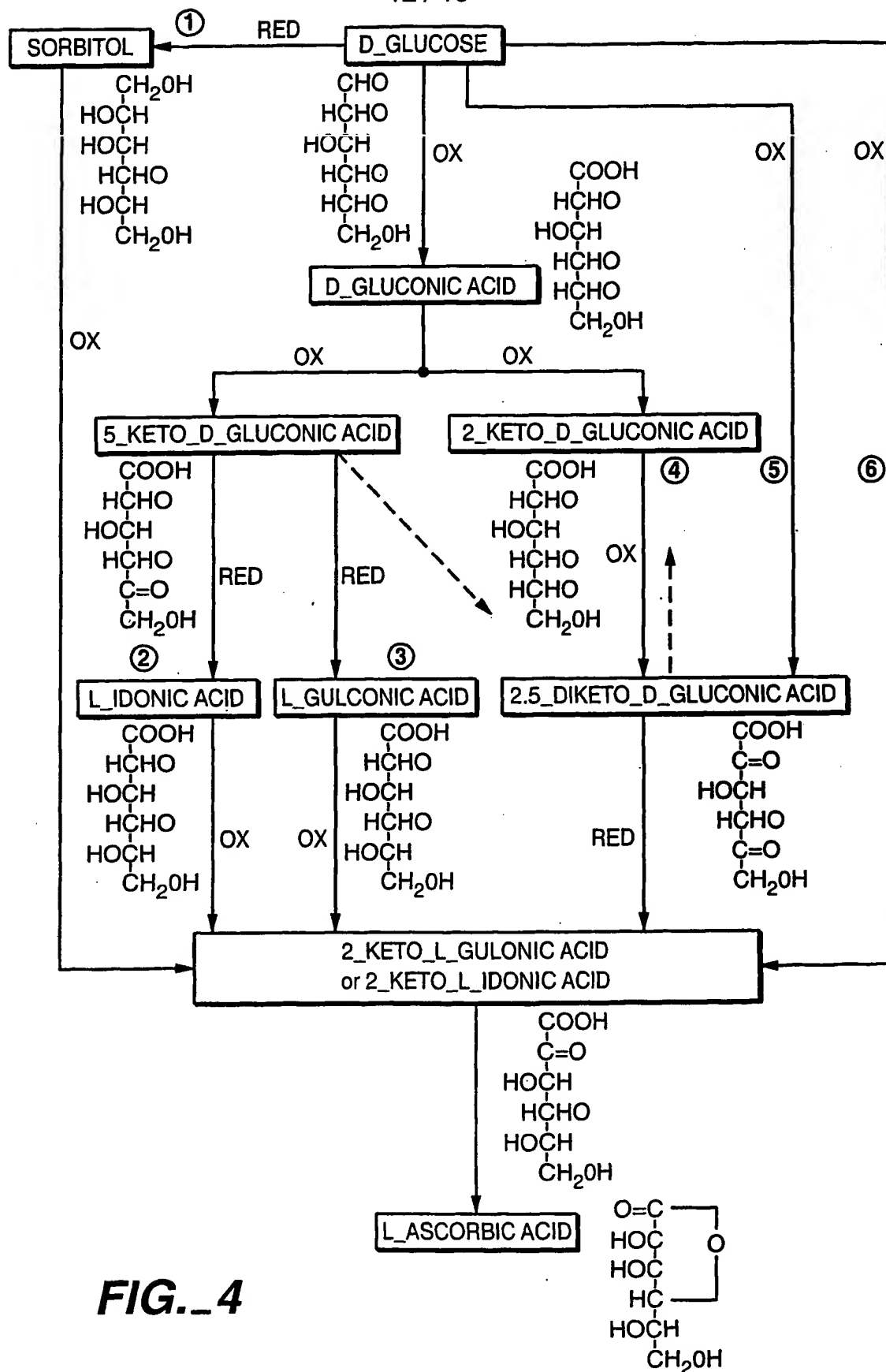
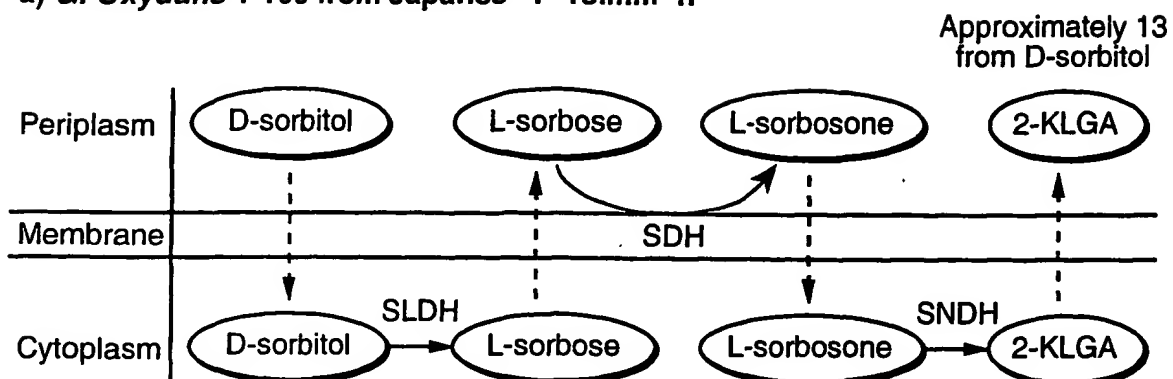
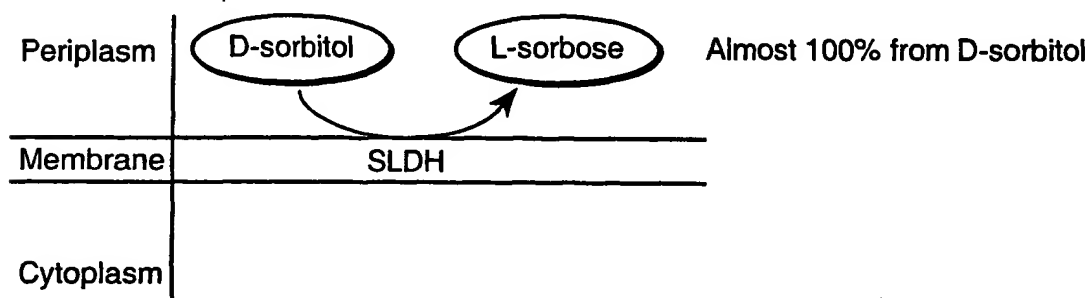
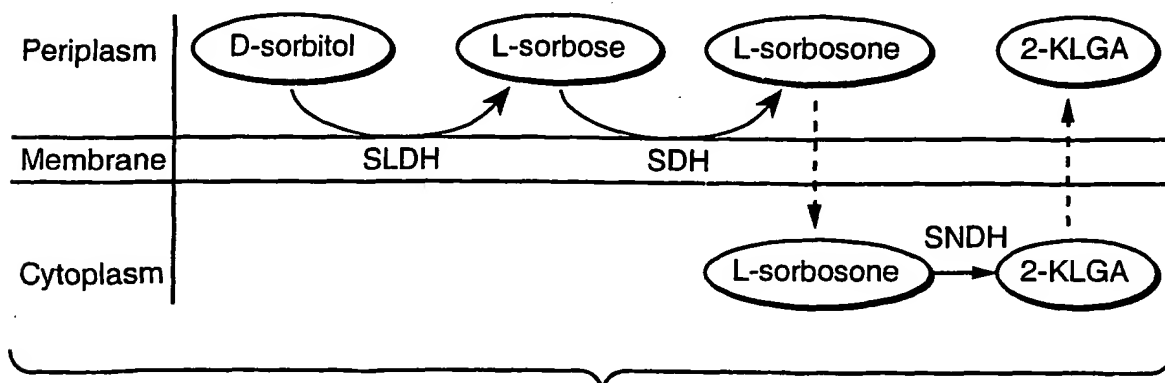
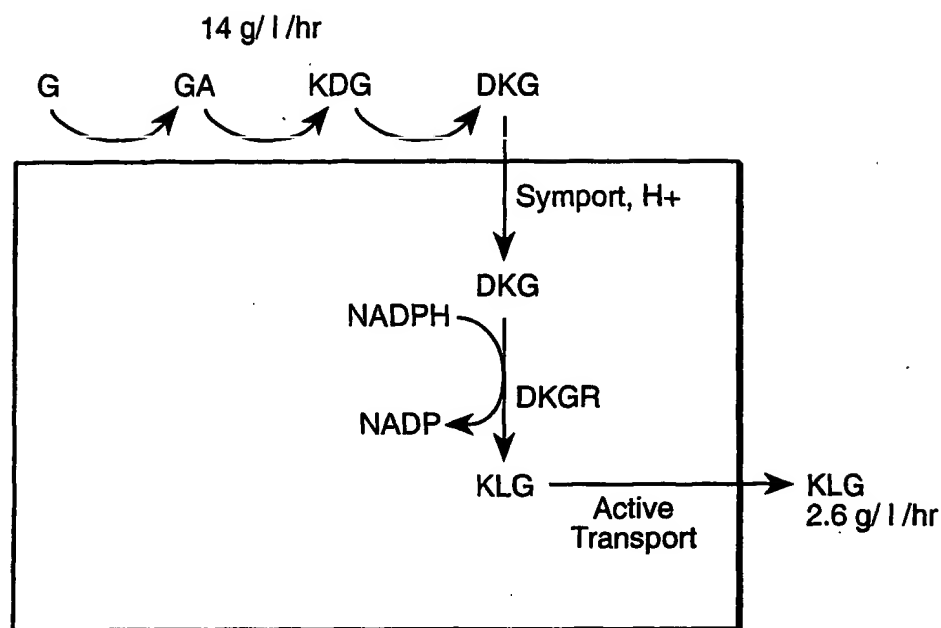


FIG.\_4

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a) *G. Oxydans* T-100 from Japanes P rsimm nb) *G. Oxydans* G624 from Japanese Peachc) Recombinant *G. Oxydans***FIG. 4A**

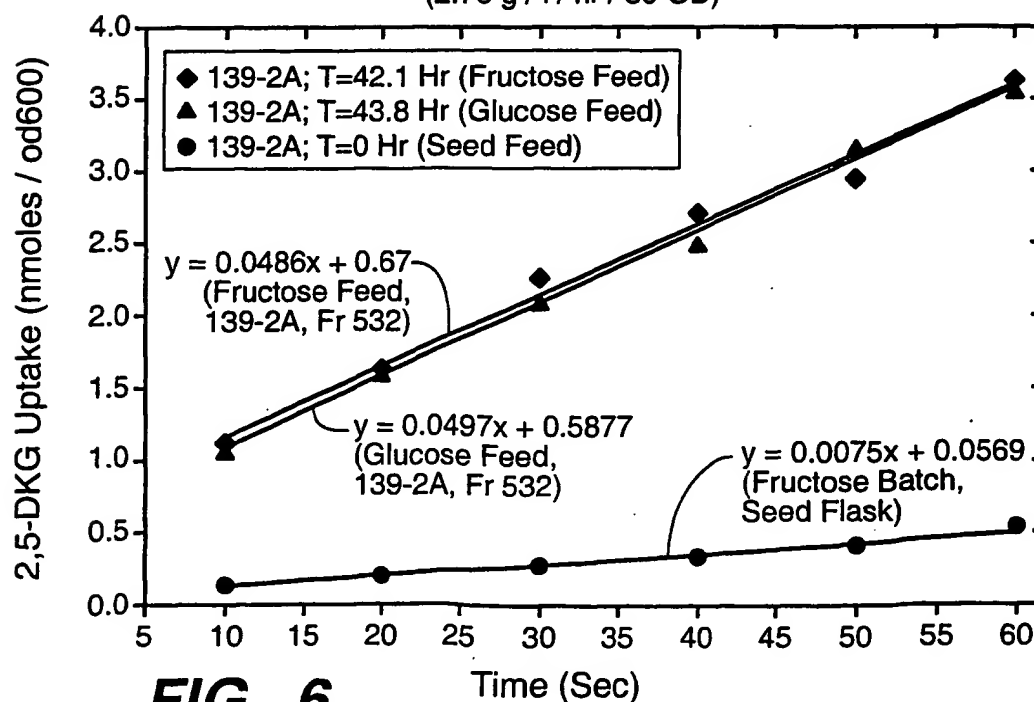
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**FIG. 5**

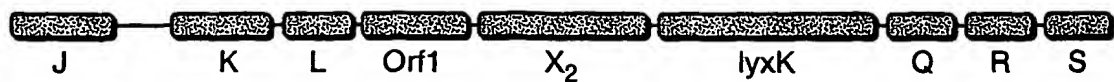
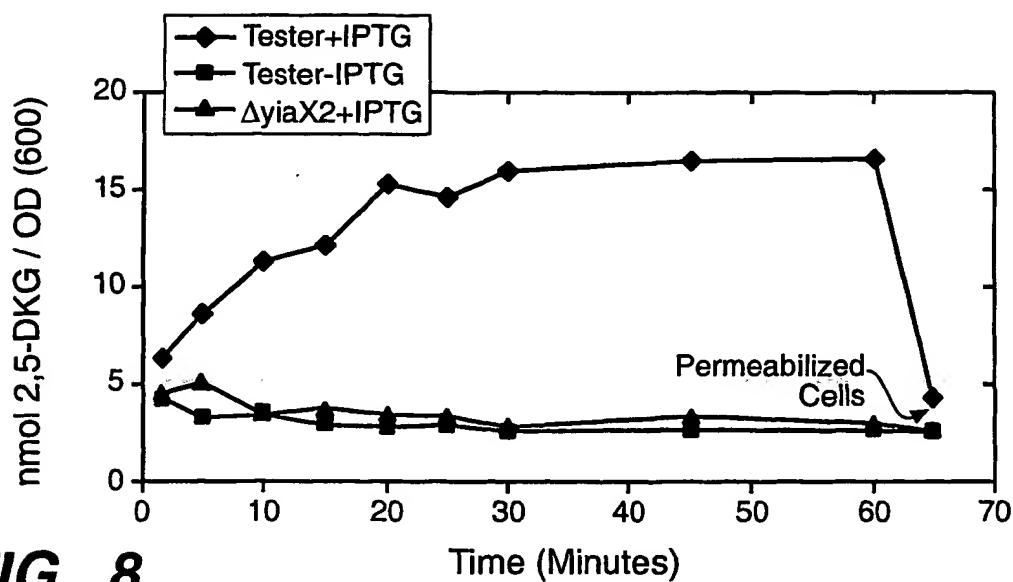
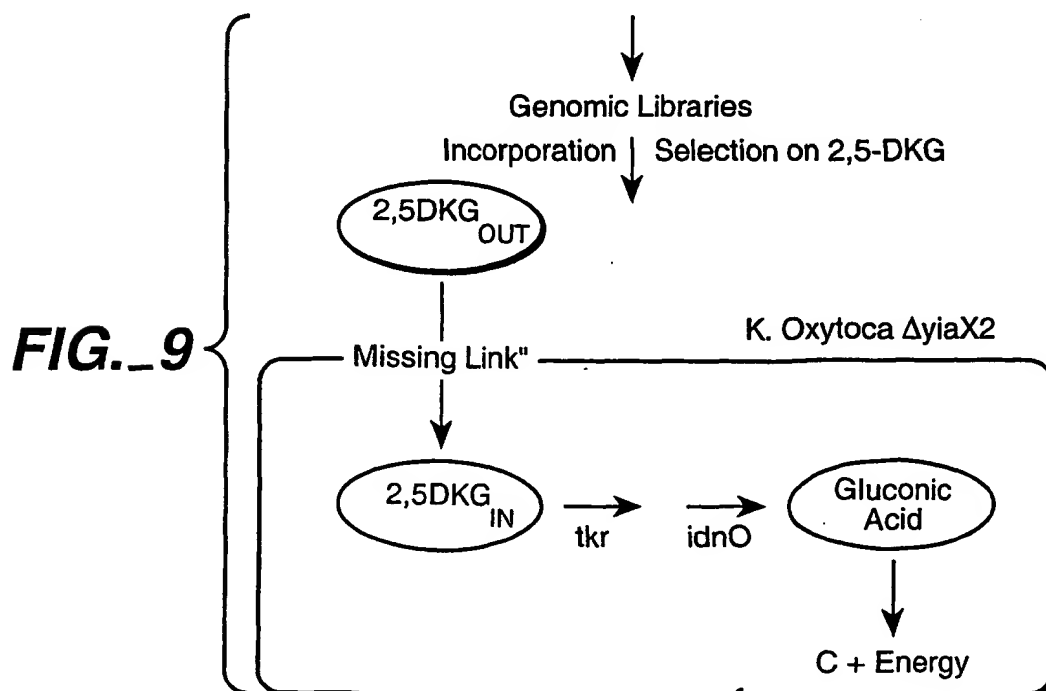
Target: 10 g / l / hr

**DKG Transport Rate Same as KLG Production Rate:  
An Inducible 2,5-DKG Permease**

DKG Transport in 2-KLG *Pantoea Citrea* Production Strain 139-2A  
(2.75 g / l / hr / 80 OD)

**FIG. 6**

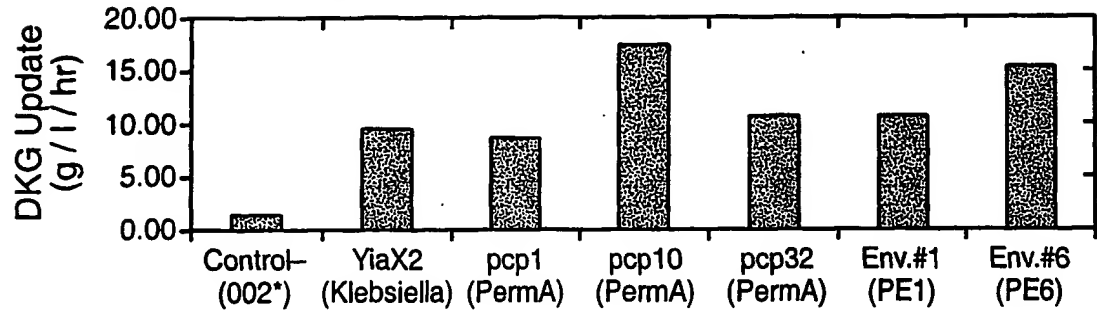
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**FIG.\_7****FIG.\_8****FIG.\_9**

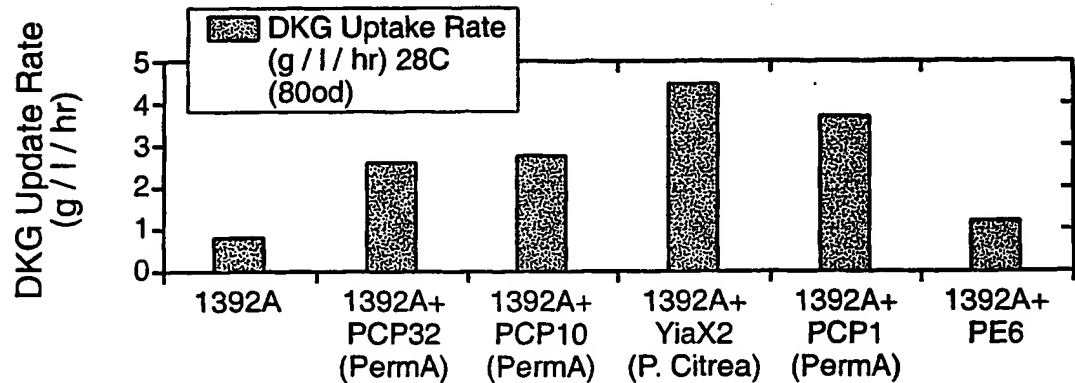
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**2,5-DKG Permeases**

DKG Uptake Activity in *K. Oxytoca* Strains  
Having Putative 2,5-DKG Permeases  
(*K. Oxytoca* 002\* Control- =  $\Delta$ yiaX2 + idno / trk)

*K. Oxytoca* Strains**FIG.\_ 10****2,5-DKG Permeases**

DKG Uptake Assay in Shake-flask Grown Cells of 139-2A / pD92  
*P. Citrea* Having Various DKG Permeases in Plasmid Vector  
Construct (pBCL1920)

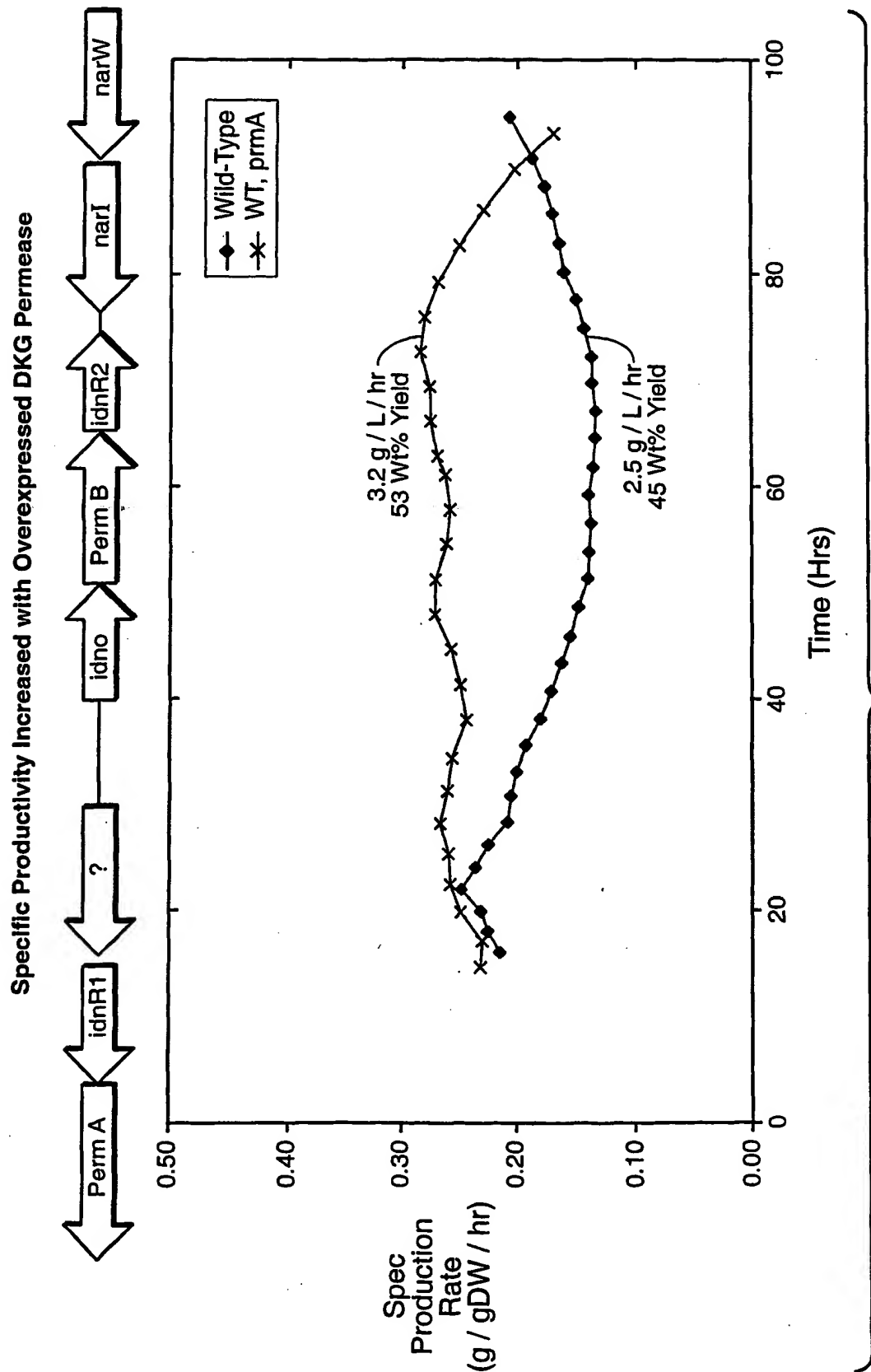
2,5-DKG Permeases Containing *P. Citrea* Strains**FIG.\_ 11****PermA Characteristics**

•Consensus: G--E---P-----W-P--ER-----

**FIG.\_ 14**

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**FIG. 12**

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## PermA Characteristics

Molecular Weight 47801.94 Daltons; 436 Amino Acids

- Average of Hydrophobicity 0.62; 9.24 Isoelectric Point
- Integral Membrane Protein with 11 Transmembrane Helices

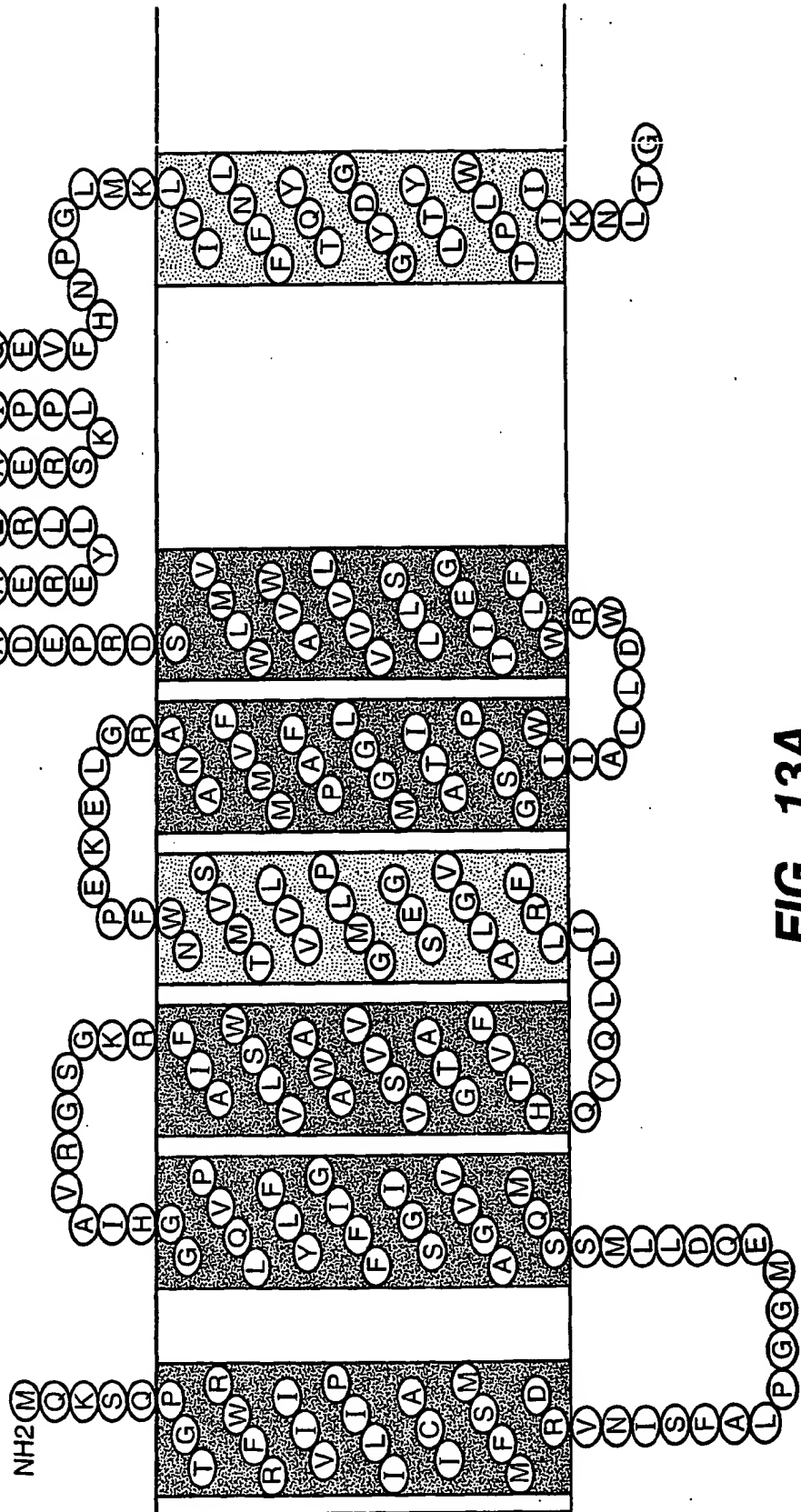


FIG. 13A

FIG. 13B

FIG. 13A    FIG. 13B

FIG. 13

